A NOVEL MAMMALIAN PIWI PROTEIN REGULATES SELF-RENEWAL AND LIFESPAN OF MACROPHAGES

DISSERTATION

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DECLARATION

I hereby declare that I completed the doctoral thesis independently based on the stated resources and aids. I have not applied for a doctoral degree elsewhere and do not have a corresponding doctoral degree. I have not submitted the doctoral thesis, or parts of it, to another academic institution and the thesis has not been accepted or rejected. I declare that I have acknowledged the Doctoral Degree Regulations which underlie the procedure of the Faculty of Life Sciences of Humboldt-Universität zu Berlin, as amended on 5th March 2015. Furthermore, I declare that no collaboration with commercial doctoral degree supervisors took place, and that the principles of Humboldt-Universität zu Berlin for ensuring good academic practice were abided by.

Marseille, 16th of April 2018

Stephanie Vargas Aguilar,

PARA FLIPITO

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ABSTRACT

PIWI proteins are the main players of an RNA-based gene regulatory machinery that represses transposable elements in the genome to prevent their mobilization and ensure genetic stability. This protective mechanism acquires particular importance in long-living, self-renewing cells, such as stem cells. PIWI proteins have thus highly conserved stem-cell functions. They are indispensable for the long-term maintenance of the somatic stem cells that drive regeneration in invertebrates, of various adult somatic stem cells in Drosophila and, most prominently, of the germline of all species studied so far. In mammals, their described functions are strictly restricted to the male germline. Despite suggestive observations for a role of PIWI proteins in the mammalian soma, robust evidence remains absent.

Similar to stem cells, tissue macrophages can locally self-renew to maintain their populations. Mechanistically, their self-renewal relies on low expression of the macrophage transcription factors MafB and cMaf, since it allows the induction of a stem cell-like network of genes that drive proliferation. Conversely, high levels of MafB and cMaf promote quiescence, because MafB directly represses the genes of the mentioned network. Accordingly, macrophages with a genetic deletion of MafB and cMaf (MafDKO macrophages) acquire the capacity to self-renew, defined by an indefinite growth in culture that does not comprise their identity and does not involve cancerogenic transformation. Similarly, macrophages with naturally low levels of MafB or cMaf, such as alveolar macrophages, display an extended self-renewal capacity in vivo and in vitro.

In this study, we have found that a short isoform of the murine Piwil2 gene, that we named 'Piwito', is expressed in MafDKO and alveolar macrophages. Piwito expression is necessary for the unaltered self-renewal of macrophages, as shown by in vitro and in vivo assays. Additionally, we show that Piwito is bound and repressed by MafB in quiescent macrophages. Interestingly, PIWI proteins in Drosophila are regulated by the MafB/cMaf homolog Traffic Jam, suggesting that the control of PIWI proteins is conserved. Finally but most importantly, we show that Piwito deficiency limits the lifespan of alveolar macrophages in culture.

This study represents the first report of a somatic function for a mammalian PIWI protein. In concordance with our observations, a recent study demonstrated a role for PIWI proteins in lifespan regulation of intestinal stem cells in Drosophila. Therfore, we propose a re-definition of the function of PIWI proteins: from a role in stem cell and genome maintenance to regulators of lifespan in self-renewing cells.

ZUSAMMENFASSUNG

PIWI Proteine sind die zentralen Darsteller eines RNA-basierten Mechanismus, der die Genregulation dient. Seine Hauptfunktion ist die Mobilisierung transponierbarer Elemente im Genom zu unterdrücken um genetische Stabilität zu gewährleisten. Dieser Schutzmechanismus ist von besonderer Bedeutung bei langlebigen, sich selbsterneuernden Zellen, wie z.B. Stammzellen. Demzufolge, sind PIWI-Proteine für die langfristige Erhaltung verschiedener Stamzellpopulationen notwendig. Beispiele dafür sind die somatischen Stammzellen, die die Regeneration bei wirbellosen Tieren vorantreiben, verschiedene adulte somatische Stammzellen in Drosophila und die Stammzellen der Keimbahn aller bisher untersuchten Tierarten. Bei Säugetieren sind die beschriebenen Funktionen von PIWI Proteinen strikt auf die männliche Keimbahn beschränkt. Trotz Andeutungen auf eine Rolle von PIWI-Proteinen in somatischen Zellen von Säugetieren, wurde eine Funktion bisher nicht beschrieben.

Ähnlich wie Stammzellen, können sich Makrophagen in verschiedenen Geweben selbst-erneuern, um ihre Populationen zu erhalten. Diese Selbsterneuerung beruht auf der geringen Expression der Transkriptionsfaktoren MafB und cMaf. Diese ermöglicht die Aktivierung eines stammzell-ähnliches Gen-Netzwerk, das die Proliferation vorantreibt. Umgekehrt fördern hohe Konzentrationen von MafB und cMaf den Austritt aus dem Zellzyklus, da MafB dieselben Gene direkt unterdrückt. Dementsprechend erwerben Makrophagen mit einer genetischen Deletion von MafB und cMaf (MafDKO-Makrophagen) die Fähigkeit zur Selbsterneuerung, definiert durch unbeschränkte Teilung in Kultur, bei der die Identität der Zellen erhalten bleibt und die keine maligne Transformation umfasst. Makrophagen mit natürlich niedriger Expression von MafB oder cMaf, wie z.B. alveoläre Makrophagen, weisen ebenso eine erweiterte Kapazität zur Selbsterneuerung auf.

In der vorliegenden Studie haben wir festgestellt, dass eine kurze Isoform des Maus-Gens Piwil2, die wir ‚Piwito' genannt haben, in MafDKO und alveolären Makrophagen exprimiert wird. Die Expression von Piwito ist für die normale Selbsterneuerung der untersuchten Makrophagen notwendig, wie die in vitro und in vivo Untersuchungen zeigen. Außerdem beweisen wir, dass Piwito von MafB in nicht-proliferierenden Makrophagen gebunden und unterdrückt wird. Interessanterweise werden PIWI Proteine in Drosophila durch den MafB/cMaf-Homologen Traffic Jam reguliert, was darauf hindeutet, dass die Kontrolle von PIWI Proteinen im Verlauf der Evolution konserviert sind. Unsere letzte aber bedeutendste Beobachtung ist dass in Ermangelung von Piwito die Lebensspane von alveolären Macrophagen in Kultur stark verkürzt ist.

Diese Studie stellt den ersten Bericht über eine somatische Funktion für PIWI Proteine in Säugetieren dar. In Übereinstimmung mit unseren Beobachtungen, zeigte eine kürzlich erschienene Studie eine Rolle für PIWI-Proteine bei der Regulation der Lebensspanne von Darm-Stammzellen in Drosophila. Wir schlagen daher eine Neudefinition der Funktion von PIWI-Proteinen vor: von einer Rolle bei der Erhaltung von Stammzellen und deren Genom zu Regulatoren der Lebensspanne in sich selbsterneuernden Zellen.

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PRELUDE

Macrophages are cellular components of the innate immune system. They are present in all multicellular organisms and are crucial contributors to various biological processes during development, homeostasis, immunity, regeneration and disease. The diversity of mechanisms they participate in reveals their plasticity and importance.

In the last years, macrophage biology has been re-evaluated at one of its most basic concepts: macrophage origin. The classical view of macrophage origin relied on a linear model in which bone marrow hematopoietic stem cells give rise to macrophage precursors, which in turn develop into blood monocytes, colonize tissues and differentiate into tissue-resident macrophages. Modern lineage tracing tools challenged that model and uncovered parallel routes of macrophage development. The concept of embryonic macrophages emerged, referring to tissue-resident macrophages that do not follow the classical developmental route via adult monocytes, but rather get settled before birth in the tissues and persist into adulthood without contribution from the bone marrow.

The discovery of the embryonic origin of macrophages turned the attention of the field towards concepts such as macrophage self-renewal or macrophage half-life. These concepts, derived from the stem cell field, shed a new light on macrophages, presenting them as cells capable of maintaining their populations, similar to stem cells. It is thus conceivable and highly interesting to ask stem cell questions for macrophages: how and when do they self-renew? Does every cell have the same self-renewal potential? How is their lifespan maintained? Do macrophages, as stem cells, age and thus get exhausted?

Macrophages that are long-lived and/or self-renewing are unavoidably subjected to the deleterious effects of time. Given enough time, all cellular processes start to fail: protein homeostasis, energy metabolism or DNA repair, just to mention a few examples. Therefore, long-living and self-renewing cells have evolved mechanisms to contain the damage caused by time and the efficiency of those mechanisms defines the length of their lifespan. Based on this reasoning, the most efficient 'time-fighting' mechanisms are the ones active in germ cells, since the germline is as close to a timeless existence as a biological system can be.

A family of RNA-binding proteins known as PIWI proteins and their associated RNA components called piRNAs are important guardians of the virtually eternal lifespan of the germline. Their main function is to repress transposable elements in the genome, thus ensuring genome stability. Throughout evolution, PIWI proteins are exclusively expressed in self-renewing cells, and their best-conserved function outside of the germline is their contribution to processes of whole body regeneration in invertebrate animals. Whole body regeneration, a process in which parts of the body can give rise to new individuals, could be considered the only other known example of a process in metazoans that comprises cells whose genome is eternal.

Part I

INTRODUCTION

The Introduction starts with a chapter on macrophage biology that briefly discusses macrophage function to then summarize the current literature on macrophage origin as well as the current knowledge on macrophage self-renewal and underlying molecular mechanisms. It also introduces alveolar macrophages, since they are a major subject of investigation in this study. The second chapter presents the concept of cellular lifespan and the limiting factors to it, with a focus on aging at the genomic level. The third and last chapter presents the two mechanisms that counteract lifespan limits that are relevant to the results of this study: sirtuins and PIWI proteins. There again, based on the results of this study, the stronger focus is on the PIWI/piRNA pathway.

1

MACROPHAGE BIOLOGY

1.1 METSCHNIKOFF AND THE DISCOVERY OF PHAGOCYTOSIS

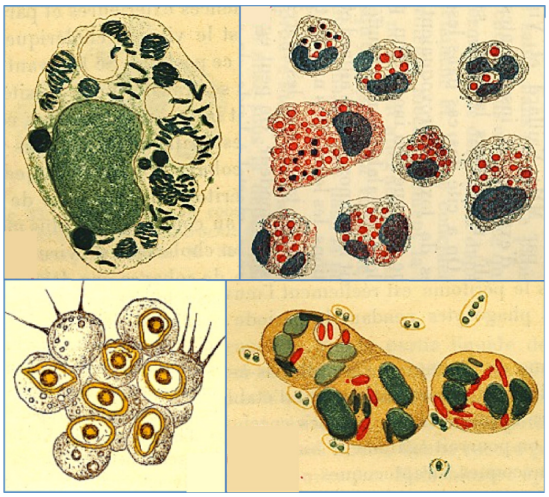

Figure I1: Drawings of phagocytes by Metschnikoff. Source: Metchnikoff (1901).

In 1883, the Russian-born scientist Ilja Iljitsch Metschnikoff wrote an essay describing his observations on "intracellular digestion" (Metschnikoff, 1883). In this essay, he was interested in the question whether primitive metazoans had specialized cells for the engulfment and digestion of food particles. As a careful observer, Metschnikoff was able to find such cells across several primitive marine organisms. He observed that they were not only capable of engulfing nutrients, but also foreign materials such as pigments or body materials such as dead cells. This essay also describes one of his most prominent experiments: he stuck rose thorns into transparent starfish larvae and saw how the same 'motile amoeboid cells' quickly started to surround the thorn. From this observation he concluded that those mysterious cells ingested particles not only for nourishment or clearing purposes, but also to protect the organism from invaders. Cellular immunity was born.

This phenomenon of cells engulfing particles or other cells was later named "phagocytosis", derived from Greek φαγειν (phagein) for "devouring" (Ambrose, 2007). Metschnikoff was not the first to witness it, but he was the first to grasp its importance in host defense against pathogens. He continued to study phagocytosis and its role in inflammation and immunity along the rest of his life. At a time where defense against pathogens was thought to be mediated exclusively by body fluids (humoral immunity), Metschnikoff proposed and became the strongest advocate of cell-mediated immunity (Cavaillon, 2011); (Figure I1).

1.2 MACROPHAGE FUNCTION

135 years after the discovery of macrophages, the number of functions they participate in continues to increase. Macrophages are evolutionary conserved, from the most primitive multicellular organisms to humans. In vertebrates, they can be found in all tissues, during all stages of development. Accordingly, macrophages participate to a wide variety of biological processes beyond immunity, such as development, tissue homeostasis, repair, and regeneration. They also play important roles in pathological processes, such as cancer, autoimmune diseases or allergies. Macrophages fulfill their functions by their ability to phagocyte, the secretion of important immunological mediators and through cellular interactions (Figure I2).

This sub-section discusses the contribution of macrophages to inflammation and tissue repair, and then presents some examples that illustrate their function in the steady state: in development and in the maintenance of tissue homeostasis

1.2.1 *Macrophages in Host Defense*

Tissue macrophages are constantly patrolling their environment. Upon encounter with a pathogen, they initiate an inflammatory response in cooperation with other local immune cells. Throughout the immune response, macrophages adapt their phenotype in concordance with the needs at the site of infection. Upon a bacterial invasion in the tissue, for example, tissue-resident macrophages sense the invader with a wide variety of receptors for the recognition of pathogen-associated molecular patterns (PAMPs) such as Toll-like receptors, NOD-like receptors or C-type lectin receptors (Taylor et al., 2005). Recognition of bacteria renders macrophages in an inflammatory phenotype. Thus, in coordination with tissue-resident mast cells, dendritic cells and stromal cells, macrophages recruit inflammatory cells from the circulation, mainly monocytes and neutrophils. Recruited monocytes differentiate into macrophages

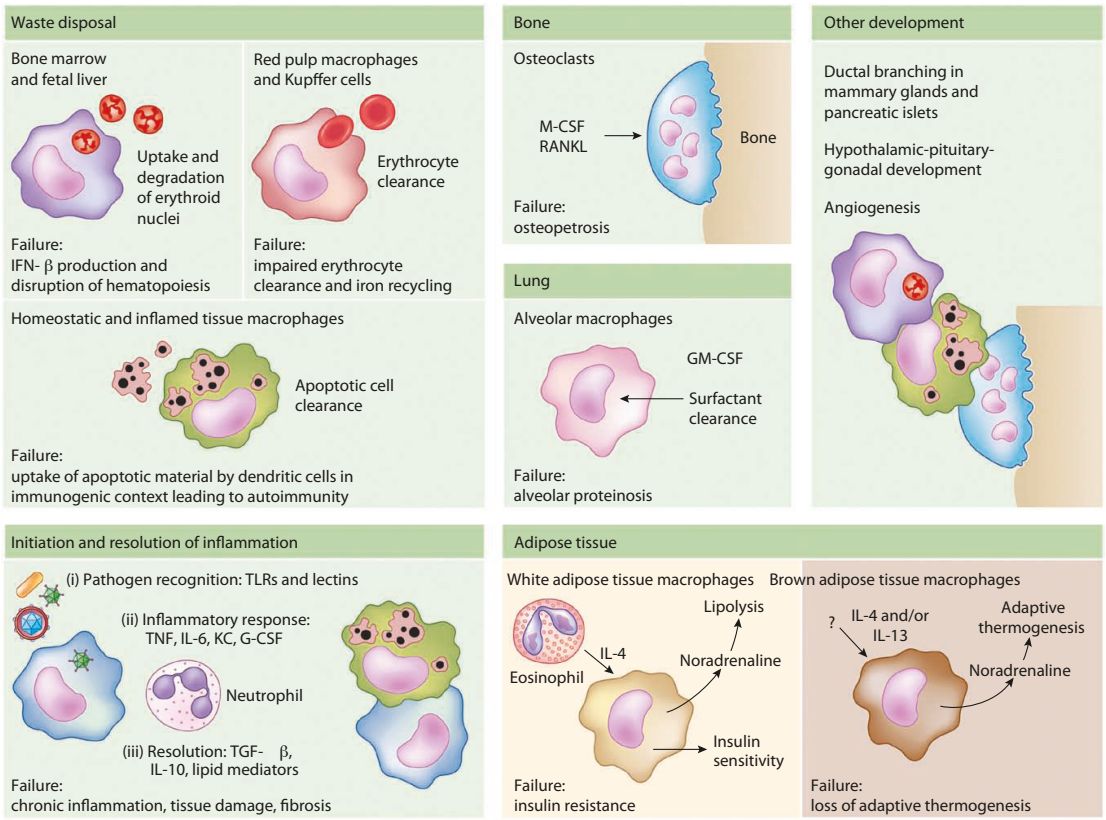

Figure I2: Macrophage function. Plasticity and importance of macrophage function exemplified by the participation of tissue-resident macrophages in highly diverse processes. Source: Davies, Jenkins, Allen and Taylor (2013).

with a pro-inflammatory phenotype as well. Inflammatory macrophages activate antimicrobial mechanisms through the secretion of pro-inflammatory mediators such as TNFα, IL1 and nitric oxide (Murray and Wynn, 2011). Subsequently and if the insult persists, they produce IL-12 and IL-23, which direct the differentiation and expansion of anti-microbial Th1 and Th17 cells to further promote the inflammatory response (Sica and Mantovani, 2012).

Reactive oxygen and nitrogen intermediates produced by pro-inflammatory macrophages, as well as the toxic activity of Th1, Th17 cells and neutrophils can cause collateral damage to the tissue. Therefore, inflammatory responses need to be resolved shortly after the causative stimulus has been eliminated. As a mechanism to terminate inflammation once it is not longer needed, pro-inflammatory macrophages undergo apoptosis or switch into an anti-inflammatory phenotype. Anti-inflammatory macrophages orchestrate the resolution of the inflammatory response by the production of immunoregulatory proteins such as IL-10, resistin-like molecule-α (RELMα), programmed death ligand 1 (PDL1) and 2 (PDL2) and arginase 1 (Murray and Wynn, 2011).

1.2.2 *Macrophages in Tissue Repair*

Upon an insult that results in tissue damage, a coordinated wound healing response is initiated by the immune system. Macrophages contribute to all steps of this response. Initially, signals from damaged cells are sensed by tissue resident macrophages and local immune cells, for example through receptors that recognize danger-associated molecular patterns (DAMPs). These activated cells initiate an inflammatory response that leads to monocyte and neutrophil influx to the site of injury. Macrophages promote the initial phases of inflammation as important sources of chemokines, matrix metalloproteases and inflammatory mediators. During and after inflammation, macrophages phagocyte cell debris and apoptotic cells derived from the tissue injury and from the inflammatory response, such as neutrophils. Removal of apoptotic inflammatory cells is a crucial step in the resolution of the inflammation.

Following the early inflammatory phase, macrophages convert into a reparative phenotype that supports tissue repair. This state is characterized by the enhanced production of growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor α (VEGF- α). These factors promote local cell proliferation and angiogenesis. Additionally, macrophages produce transforming growth factor $\beta 1$ (TGF-$\beta 1$), which stimulates local and recruited fibroblasts to differentiate into myofibroblasts to facilitate the wound contraction and closure. Myofibroblasts and macrophages coordinate the restoration and remodeling of the extracellular matrix. In the final stages of wound healing, macrophages adopt and anti-inflammatory phenotype and secrete immune-modulatory factors to contribute to the resolution of the inflammation (Vannella and Wynn, 2017).

1.2.3 *Macrophages in Development*

Macrophages are indispensable sculptors and guides of a developing organism. Macrophage depletion comprises several processes during development, including the branching morphogenesis of the pancreas or the mammary gland, neural patterning in the brain, angiogenesis, bone morphogenesis or the development of adipose tissue (Pollard, 2009; Wynn et al., 2013). As examples, the absence of osteoclasts (bone-reabsorbing macrophages) results in voluminous, non-sculpted bones that lack the cavities needed for adult hematopoiesis (Wiktor-Jedrzejczak et al., 1991) and microglia deficiency during development leads to brain structural defects in the adulthood (Erblich et al., 2011).

Frequently, macrophages fulfill their functions during development by providing crucial growth factors in the right place and at the right time. One remarkable example is the role of macrophages in vessel patterning. Studies

have shown that macrophages, as sources of pro- and anti-angiogenic factors, guide the formation of vessel networks (Wynn et al., 2013). More specifically, macrophages in the retina can initiate non-canonical WNT signaling to fine-tune the branches of the retinal vascular plexus (Stefater III et al., 2011).

Finally, macrophages also contribute to development through phagocytosis of apoptotic cells. As an example, microglia engulf and eliminate synaptic material in the postnatal mouse brain. This synapse pruning ensures the correct maturation of brain circuits (Paolicelli et al., 2011).

1.2.4 *Macrophages in Homeostasis*

Tissue macrophages contribute to the maintenance of homeostasis by constantly patrolling their surroundings in the search for tissue damage or intruders. They remove dead cells, debris or toxic materials. According to the tissue they reside in, their functions in the maintenance of homeostasis may differ: alveolar macrophages participate in the removal of excess mucus from the alveoli to ensure free airways for gas exchange. Resident macrophages of the spleen red pulp and the liver ingest and digest damaged or senescent erythrocytes, releasing hemoglobin that is then recycled to maintain iron homeostasis (Davis et al., 2013). During the production of red blood cells, macrophages in the bone marrow or fetal surround the maturing erythroblasts to ingest their expelled nuclei. The clearance of these nuclei is crucial, since erythropoiesis is blocked in the absence of macrophages (Kawane et al., 2001; Yoshida et al., 2005).

As for all other macrophage functions, macrophages do not participate in homeostasis only through phagocytosis. In the vasculature, for example, macrophages help to decrease systemic blood pressure upon salt uptake by secreting vascular endothelial growth factor (VEGF-C). This growth factor expands lymphatic capacity and drains fluid, relieving pressure from the blood circulatory system (Machnik et al., 2009).

An example of a remarkable and non-classical function of macrophages in homeostasis is given in the heart: it has been shown that cardiac macrophages facilitate electrical conduction through the atrioventricular node of the heart, the area that electrically connects the atria and the ventricles at the base of the right atrium. Conduction is supported by gap junctions that electrically couple macrophages to cardiomyocytes (Hulsmans et al., 2017).

1.3 MACROPHAGE ORIGIN

1.3.1 *Developmental Pathways of Tissue-Resident Macrophages*

Hematopoiesis occurs in three successive developmental waves. Soon after gastrulation, the first hematopoietic site arises in the extra-embryonic yolk sac. It comprises cellular aggregates of erythromyeloid progenitors (EMPs), named "blood islands", that give rise to cells from the erythroid and myeloid lineage exclusively (Moore and Metcalf, 1970; Palis et al., 1999). Macrophages derived from this 'primitive hematopoiesis' have a unique differentiation pattern in the sense that they arise directly from progenitors and do not pass through a monocytic intermediate as it is seen in macrophages derived from later waves of hematopoiesis. Once the circulatory system in the embryo is established, yolk sac-derived macrophages spread throughout the embryo and colonize the organs. Similarly, EMPs migrate to the fetal liver, where they transiently sustain the production of fetal erythrocytes, macrophages, granulocytes and monocytes. Among the EMP-derived populations, only macrophages persist into adulthood (Perdiguero et al., 2015).

Shortly after the appearance of the first hematopoietic cells in the yolk sac, hematopoietic stem cells with multi-lineage potential arise in the aorta, gonads and mesonephros (AGM) region of the embryo (Medvinsky et al., 1993). After generation in the AGM, hematopoietic stem cells migrate to the fetal liver and establish the 'definitive hematopoiesis' (Kumaravelu et al., 2002). The fetal liver becomes then the principal site for hematopoiesis in the embryo, generating all hematopoietic lineages including monocytes (Naito et al., 1990). Monocytes are recruited to all embryonic tissues, where they can differentiate to tissue-resident macrophages and cohabit with yolk sac-derived macrophages. These two populations, termed from now on collectively as 'embryonic macrophages', can persist in the colonized organs during the whole life of an organism, maintaining their populations through local proliferation (Schulz et al., 2012; Yona et al., 2013; Hashimoto et al., 2013; Perdiguero et al., 2015; Hagemeyer et al., 2016; Mass et al., 2016).

Around the perinatal period, the bone marrow replaces the fetal liver as the main organ for definitive hematopoiesis (Orkin and Zon, 2008). Hematopoietic stem cells persist in the bone marrow throughout adulthood and remain the source of cells for all hematopoietic lineages. Monocytes derived from this adult definitive hematopoiesis continue to enter and settle in tissues throughout life.

1.3.2 *Heterogeneity of Tissue-Resident Macrophages*

Tissue-derived macrophages can have thus a mixed origin and different lifespan. Genetic fate mapping, bone marrow transplantation and parabiosis studies have proven that the individual contribution of macrophages from different origins to a given tissue macrophage pool is highly variable (Figure I3).

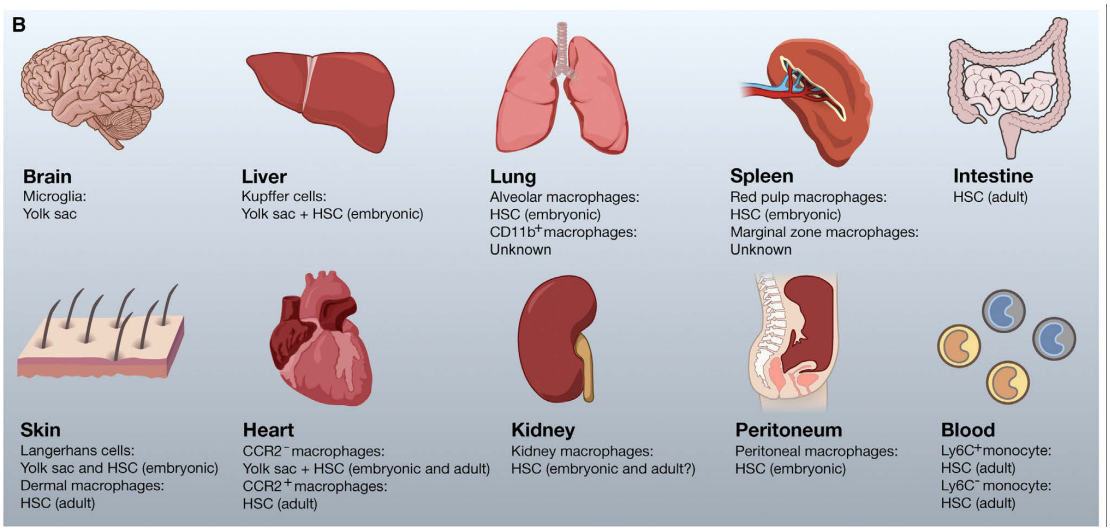

Figure I3: Heterogeneity of tissue-resident macrophages. Most adult tissue-resident macrophages are of mixed origin. They can be derived from yolk sac embryonic myeloid progenitors, from embryonic hematopoietic stem cells (HSC) in the fetal liver or from adult HSC in the bone marrow. Adapted from Epelman, Lavine and Randolph (2014).

The resident macrophage population of the brain, the microglia, is exclusively yolk-sac derived with no contribution from monocytes in the steady state (Ajami et al., 2007; Ginhoux et al., 2010; Kierdorf et al., 2013). Langerhans cell in the skin epidermis, which are considered as macrophages due to their macrophage colony-stimulating factor (M-CSF) dependence (Satpathy et al., 2012), are also exclusively of embryonic origin. However, in this case, the population is a mixture of yolk sac-derived and fetal monocyte-derived macrophages (Merad et al., 2002; Chorro et al., 2009; Hoeffel et al., 2012).

Several studies have shown that tissue-resident populations in organs such as the liver, lung, spleen and peritoneum have a prenatal origin and persist into adulthood with little or no contribution of monocytes (Schulz et al., 2012; Yona et al., 2013; Hashimoto et al., 2013; Perdiguero et al., 2015). By contrast, some tissue macrophage populations have been reported to depend almost exclusively on bone marrow hematopoiesis during adulthood, such as the tissue-resident macrophages in the adult intestinal lamina propria (Zigmond et al., 2012; Bain et al., 2014) or in the dermis (Jakubzick et al., 2013; Tamoutounour et al., 2013).

In a tissue- and context-specific manner, origin heterogeneity can also be a dynamic process. In tissues such as the heart (Molawi et al., 2014) or the testis (Mossadegh-Keller et al., 2017), embryonic macrophages get replaced by circulating monocytes over time in the steady state. This replacement of embryonic macrophages by monocyte-derived macrophages becomes more apparent upon an insult: cardiac macrophages get replaced with faster dynamics under challenge conditions, such as inflammation or experimental depletion (Epelman et al. 2014). In the same manner, embryonic-derived Kupffer cells (liver resident macrophages) get depleted during a systemic bacterial infection, and recruited monocytes regenerate the liver-resident macrophage pool once the infection was resolved. Interestingly, monocytes repopulate the empty liver niche by local proliferation, rather than by further recruitment of circulating monocytes (Blériot et al., 2015). This observation emphasizes the importance of local macrophage self-renewal for the maintenance of most adult tissue macrophage populations, irrespectively of their origin.

1.4 MACROPHAGE SELF-RENEWAL

As outlined in section 1.3 about macrophage origin, local macrophage proliferation is a major mechanism to maintain constant tissue macrophage numbers in adulthood, irrespectively of their origin. Importantly, it does not only maintain macrophage populations in the steady state (Chorro et al., 2009; Hashimoto et al., 2013), but it also causes the expansion of macrophages in a non-physiological context, such as a chronic inflammation. In this regard, it has been shown that macrophages accumulate by local proliferation in the arterial walls during atherosclerosis (Robbins et al., 2013), similarly to adipose tissue macrophages during obesity (Haase et al., 2013; Amano et al., 2014; Zheng et al., 2016). Local proliferation also serves as an emergency mechanism to replenish macrophage populations that have been depleted, either in a pathological situation such as infection (Blériot et al., 2015) or experimentally (Davies et al., 2011; Hashimoto et al., 2013). Finally, local macrophage proliferation has been also suggested to directly contribute to host defense, for example by ensuring a quick and local expansion of monocyte-derived macrophages at the site of injury during a parasitic infection (Jenkins et al., 2011, 2013).

However, whether this proliferation can be considered self-renewal is still debated. Self-renewal is defined as the process by which a cell divides to generate more cells of the same identity, with the purpose of perpetuating a given cell population. In this sense, it is unclear whether resident macrophages are replenished from local progenitors or whether mature cells of the population are capable of cell divisions that generate new cells of the same identity. Only the latter scenario would indicate maintenance of the population by self-renewal of mature cells. One study shows that microglia arise from a local committed progenitor in steady state (Elmore et al., 2014). However, there are several reports

of examples where fully differentiated macrophages re-enter the cell cycle to perpetuate their populations in vivo (Bouwens et al., 1986; Lawson et al., 1992; Chorro et al., 2009; Davies et al., 2011; Kanitakis et al., 2010; Hashimoto et al., 2013; Soucie et al., 2016). Additionally, proliferating macrophages conserve surface markers of mature macrophages such as F4/80 (Lawson et al., 1992; Soucie et al., 2016; Aziz et al., 2009) and mature macrophage functions such as phagocytosis (Aziz et al., 2009). Finally, one study indicated that alveolar macrophages proliferate in a stochastic manner after experimental depletion, regardless of whether they have divided before or not (Hashimoto et al., 2013). Therefore, current literature supports the idea that macrophages self-renew.

1.4.1 *Extracellular Inducers of Macrophage Self-Renewal*

Macrophage Colony-Stimulating Factor and Interleukin 34

Macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor 1 (Csf1), is the main regulator of differentiation, proliferation and survival of monocytes, macrophages and some dendritic cell subtypes (Stanley et al., 1997; Chitu and Stanley, 2006; Hamilton, 2008). Its name derives from the observation that it promotes the growth of macrophage colonies from bone marrow progenitors (Stanley et al., 1978). Mice and rats with natural mutations that impair the M-CSF gene have a generalized decrease of tissue macrophages, along with severe phenotypes associated with missing macrophage functions (Yoshida et al., 1990; Wiktor-Jedrzejczak et al., 1991; Van Wesenbeeck et al., 2002).

Macrophages in culture enter the S-phase of the cell cycle upon M-CSF simulation (Tushinski and Stanley, 1985; Roussel, 1997). Consistently, several studies have shown that M-CSF is able to promote macrophage proliferation in vivo. Macrophage self-renewal in the peritoneal cavity is M-CSF dependent in the steady state and upon inflammation (Davies et al., 2013, NC; Jenkins et al. 2013). M-CSF also drives an expansion of uterine macrophages during pregnancy (Tagliani et al., 2011). More generally, it has been shown that upon experimental depletion, the resident macrophages of the spleen, peritoneal cavity and lung repopulate the empty niches under the stimulation of M-CSF (Hashimoto et al., 2013).

M-CSF acts through its receptor M-CSFR (also known as CSF1R or CD115). However, mice with mutations that impair the M-CSFR have a more severe phenotype mice lacking M-CSF. Notably, microglia and Langerhans cells are present in M-CSF deficient mice, but absent in M-CSFR deficient mice (Witmer-Pack et al., 1993; Ginhoux et al., 2006, 2010). This is explained by the fact that M-CSFR has an alternative ligand: interleukin 34 (IL-34), another important cytokine in the control of macrophage self-renewal. IL-34 is abundantly pro-

duced by neurons and keratinocytes and it contributes to the establishment and maintenance of microglia and Langerhans cells (Wang et al., 2012; Greter et al., 2012).

Granulocyte-Macrophage Colony-Stimulating Factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a further crucial growth factor for macrophage establishment and maintenance. GM-CSF is indispensable for the development and self-renewal of alveolar macrophages in the lung (Shibata et al., 2001), not only in the steady state, but also after lethal irradiation (Hashimoto et al., 2013). Its effects are not restricted to alveolar macrophages, since it has been shown to promote the proliferation of macrophages in the omentum (Koenen et al., 1996). The effect of GM-CSF on macrophage proliferation has also been shown in vitro, where it can sustain the long-term culture of alveolar macrophages (Soucie et al., 2016). Additionally, two studies have shown that macrophage-like cell lines derived from the fetal liver (Fejer et al., 2013) or adult bone marrow (Ito et al., 2013) can be maintained in culture in the presence of GM-CSF. However, in both cases, it is not known whether these cell lines are derived from a progenitor and thus remain in an immature state. Furthermore cell populations from GM-CSF cultures derived from complex cell sources, such the bone marrow or the fetal liver, appear to be heterogeneous (Helft et al., 2015).

Interleukin 4

The cytokine interleukin 4 (IL-4) is emerging as a potent inducer of macrophage proliferation. IL-4 promotes T helper type 2 (Th2) immune responses and polarization of macrophages towards an anti-inflammatory (or alternatively activated) phenotype (Murray and Wynn, 2011). Unexpectedly, it was shown that during a typical Th2 immune response, a nematode infection in the pleural cavity, alternative activated macrophages were not recruited from the blood to the infection site, but rather derived from local proliferation of tissue-resident macrophages (Jenkins et al., 2011). This proliferation was a crucial step in the immune response and was mediated by IL-4. The authors could also show that exogenous IL-4 delivered intra-peritoneally was sufficient to induce macrophage proliferation in vivo, outside of the context of an infection. Interestingly, the effect was not limited to resident peritoneal macrophages, but could also be seen in recruited monocytes, Kupffer cells and macrophages in the pleural cavity. Of note, experiments with IL-4 receptor alpha chain (IL-4Rα)-deficient macrophages demonstrated that IL-4 acts directly on macrophages to induce proliferation (Jenkins et al., 2013).

IL-4-driven proliferation has not been shown so far in the context of steady state proliferation. It is therefore possible that IL-4 drives macrophage proliferation only under challenge conditions. This idea is supported by the observation that steady state proliferation of the pleural cavity and peritoneal macrophages is not affected in the absence of IL-4 signaling and also by the fact that Th2 cells are necessary for macrophage proliferation during a parasitic infection (Loke et al., 2007; Jenkins et al., 2013). Th2 cells may be an important source of IL-4 during infection, allowing proliferation of macrophages beyond steady-state (which is mostly M-CSF-dependent) levels and keeping this extended proliferation localized to the infection site. Importantly, however, there is also a CSF1R-dependent component to macrophage proliferation during nematode infection (Jenkins et al., 2013).

1.4.2 *Intracellular Mechanisms of Macrophage Self-Renewal*

Signaling Pathways of Macrophage Self-Renewal

M-CSF binds and activates the receptor tyrosine kinase M-CSFR. Binding of the ligand results in receptor dimerization and auto-phosphorylation of tyrosine residues on the cytoplasmic domains of the receptor dimers. This initiates a cascade of phosphorylation that activates numerous kinases, which in turn guide the signal transduction through different signaling pathways to orchestrate a given cellular outcome, such as proliferation, differentiation or survival (Hamilton, 1997; Yeung and Stanley, 2003). A series of in vitro studies have elucidated which of the various pathways activated upon M-CSF stimulation are responsible for macrophage proliferation, pointing out pathways such as the MAPK/ERK pathway, the SFK pathway or the PI3K-Akt pathway (Jaworowski et al., 1999; Valledor et al., 2000; Munugalavadla et al., 2005; Takeshita et al., 2007; Yu et al., 2012). This mitogenic response leads to the expression of early (such as c-fos, c-jun, c-myc, Ets1 or Ets2) and late (e.g. D-type cyclins) cell-clycle genes, which then coordinate the initiation and progression of the cell cycle (Roussel, 1997).

Interestingly, it has also been shown that the transmembrane adaptor protein DAP12 mediates M-CSFR proliferative signals independently of MAPK or Akt signaling. Upon M-CSFR activation, the cytoplasmic domain of DAP12 initiates a phosphorylation cascade that ends with the phosphorylation of β-catenin, triggering its nuclear translocation and the activation of pro-proliferative genes, such as cyclin D1 and c-Myc. DAP 12 deficiency specifically affects proliferation and survival, leaving differentiation untouched (Otero et al., 2009).

The GM-CSF receptor is a heterodimer composed by a major binding subunit (GMRα) and a major signaling subunit (βc) (Hayashida et al., 1990). Im-

portantly, the βc subunit is shared by the closely related IL-3 and IL-5 receptors. In contrast to the M-CSF receptor, the GM-CSF receptor does not have an intrinsic tyrosine kinase activity. Upon ligand binding, the βc subunit associates with the tyrosine kinase Jak2, which in turn transphosphorylates the βc subunit to initiate a cellular response (Guthridge et al., 1998). Several signaling pathways get activated upon GM-CSF stimulation. One study has shown that shown a role for the mechanistic target of rapamycin (mTOR) pathway in the GM-CSF dependent proliferation and maintenance of alveolar macrophages (Deng et al., 2016). Additionally, it has been shown that STAT5 is activated upon GM-CSF signaling and that it is required and sufficient for macrophage proliferation (Feldman et al., 1997; Fejer et al., 2013).

Similarly, little is known about the signaling pathways promoting macrophage self-renewal upon IL-4 activation. There, the PI3K-Akt pathway has been shown to mediate IL-4 dependent macrophage proliferation (Ruckerl et al., 2012).

Gene Expression During Macrophage Self-Renewal

Mechanistically, our laboratory has shown that proliferating macrophages activate a set of self-renewal genes that are shared with embryonic stem (ES) cells. These genes are organized as a network and influence each other's expression. The network has Myc and Klf2 as central nodes and genes such as Klf4, Chd1, Nfya and Stat3 as prominent members. Interestingly, the study showed that macrophages and embryonic stem cells activate those genes using a distinct set of enhancers.

Genome-wide enhancer usage, i.e. which enhancers are active, poised or inactive, is a definition of cell identity. Macrophages access the self-renewal gene network through macrophage-specific enhancers, whereas ES cells utilize ES cell-specific enhancers to activate the same group of genes. Hence, this study proposes an interesting genomic mechanism on how ES-cell like self-renewal could be compatible with the maintenance of macrophage identity (Soucie et al., 2016).

Furthermore, the study showed that the transcription factor MafB directly represses the self-renewal network, therefore inhibiting macrophage proliferation. This observation indicates that self-renewal is a reversible process, globally kept under control by transcription factors such as MafB. In line with this, the study showed that macrophages with a deletion of MafB and cMaf, as well as macrophages with naturally low levels of these two transcription factors, such as alveolar macrophages, are able to activate the self-renewal genes and thus proliferate. Furthermore, MafB regulation of macrophage self-renewal may be also be relevant in vivo, since macrophages in the peritoneum, liver and spleen did not express MafB while cycling and proliferative stim-

uli such as M-CSF temporally down-regulated MafB expression (Soucie et al., 2016).

MafB and c-Maf in the Control of Macrophage Self-Renewal

Maf transcription factors belong to the activating protein-1 (AP-1) superfamily of transcription factors and can be classified into large and small Mafs. MafA, MafB, cMaf and NRL are large Maf transcription factors. They have a highly conserved basic region leucine zipper (bZip) structure. The basic domain serves the DNA interaction since it recognizes and binds a long DNA sequence known as the Maf recognition element (MARE). The leucine zipper domain allows the formation of homo- or heterodimers between mutually compatible bZip transcription factors, like Fos and Jun (Kataoka et al., 1994; Kerppola and Curran, 1994). Additionally, Maf transcription factors can interact with unrelated transcription factors like Ets family members (Sieweke et al., 1996). Maf transcription factors can thus influence gene expression by two mechanisms: direct DNA binding or through interaction with other transcription factors.

MafB and c-Maf have been classified as core macrophage transcription factors (Gautier et al., 2012). In the hematopoietic system, MafB expression is strongly upregulated upon macrophage differentiation and is largely specific to the myeloid lineage. This expression pattern is conserved across species (Sieweke et al., 1996; Eichmann et al., 1997; Kelly et al., 2000; Hamada et al., 2003; Gemelli et al., 2006) and suggests an important role for MafB during macrophage differentiation. Several observations support this notion, such as the fact that overexpression of MafB in human (Gemelli et al., 2006) and chicken (Kelly et al., 2000) hematopoietic progenitors induces increased myeloid commitment, whereas the expression of a dominant negative form of MafB inhibits myeloid colony formation and macrophage differentiation (Kelly et al., 2000). Several studies indicate that MafB can promote macrophage fate at the expense of other cell fate options by directly interacting with key transcription factors: it binds and represses the transcription factor Ets-1 to inhibit erythroid differentiation (Sieweke et al., 1996) and counteracts low levels of PU.1 to block dendritic cell fate (Bakri et al., 2005). Mechanistically, MafB could drive myeloid differentiation indirectly by regulating the cell cycle. In line with this hypothesis, it has been shown that MafB binds and represses Myb in mouse myeloid progenitors to block their expansion and promote differentiation (Tillmanns et al., 2007).

c-Maf is closely related to MafB with regard to its expression patterns, interaction partners and functions (Kataoka et al., 1994; Hedge et al., 1998). As MafB, c-Maf interacts with Myb in immature human myeloid cell lines to promote their differentiation towards the myeloid lineage (Hedge et al., 1998). Consistently, its overexpression in human hematopoietic progenitors promotes

monocyte/macrophage differentiation (Gemelli et al., 2006). This suggests that both Maf transcription factors have overlapping, redundant functions in the myeloid system.

Surprisingly, however, macrophages develop efficiently in the absence of MafB (Aziz et al., 2006) or in the combined absence of MafB and cMaf (Aziz et al., 2009). They are present in normal numbers, show markers of mature macrophage and are able to perform mature macrophage functions such as phagocytosis (Aziz et al., 2006, 2009). Nevertheless, MafB-/- macrophages display a different actin organization upon M-CSF stimulation when compared to their WT counterparts, involving a rapid formation of filopodial and strongly branched protrusions (Aziz et al., 2006).

On the other hand, macrophages deficient for MafB and cMaf (MafDKO macrophages) display the astonishing capacity to proliferate indefinitely upon M-CSF stimulation, without loosing their differentiated identity. Of note, this proliferation is not tumorigenic (Aziz et al., 2009). This supports the notion that MafB is involved in cell cycle control, not only of myeloid progenitors, but also of differentiated macrophages. Consistently, our laboratory has shown that MafB regulates the proliferation of macrophage populations in vivo as well, demonstrated in the example of alveolar and peritoneal macrophages, as well as macrophages in the spleen and liver (Soucie et al., 2016). Furthermore, another study from our laboratory showed that MafB limits asymmetric myeloid commitment divisions of hematopoietic stem cells (HSCs) (Sarrazin et al., 2009), providing an example of MafB-dependent control of the cell cycle during myelopoiesis.

1.4.3 *MafDKO Macrophages as a Tool to Study Macrophage Self-Renewal*

As stated above, deficiency of the transcription factors MafB and cMaf confers macrophages with the ability to self-renew indefinitely without tumorigenic transformation. Macrophages deficient for MafB and cMaf (MafDKO macrophages) express mature macrophage surface markers such as CD11b, F4/80 and CD115 and are negative for progenitor markers such as CD117 and CD34. Consistently, MafDKO macrophages and WT counterparts display a highly similar profile in global gene expression analysis (Aziz et al., 2009).

Regarding macrophage function, MafDKO macrophages are able to fulfill macrophage functions: they produce nitric oxide in response to lipopolysaccharide (LPS) or interferon-γ (IFN γ) and are able to phagocyte beads or living bacteria in vitro. Importantly, their ability to phagocyte living bacteria is conserved in MafDKO macrophages expressing the proliferation marker Ki67, suggesting that mature macrophage identity and function are maintained throughout the cell cycle. In vivo, MafDKO macrophages contribute to tissue macro-

phage populations of the bone marrow, peritoneum, spleen and liver. Finally, as suggested by results in vitro, MafDKO macrophages are able to participate in infection processes in vivo by phagocytosis of bacteria and the production of inducible nitric oxide synthase (iNOS) (Aziz et al., 2009). As outlined above, the self-renewal of MafDKO macrophages relies on the upregulation of a self-renewal network that is shared with ES cells (Soucie et al., 2016).

Taken together, MafDKO macrophages are fully functional mature macrophages that allow the study of macrophage self-renewal and its regulation by the transcription factor MafB and cMaf. They are easily grown in culture and, most importantly, they use mechanisms of macrophage proliferation that are relevant in vivo (Soucie et al., 2016).

1.5 ALVEOLAR MACROPHAGES

1.5.1 *Identification of Alveolar Macrophages*

Alveolar macrophages (AM) are one of the resident macrophage populations of the lung. They populate the airway lumina, also known as alveolar space or alveoli, where they account for approximately 95% of the cellular content in steady state. As opposed to most other macrophage populations that depend on the growth factor M-CSF for their establishment and survival, alveolar macrophages depend on GM-CSF.

Due to their GM-CSF dependency and the fact that they reside in a niche that is strongly exposed to the external environment, alveolar macrophages display a distinct macrophage phenotype. In steady state, they express classical macrophage markers, such as MerTK, CD64 and F4/80 (Gautier et al., 2012), very high levels of the integrin CD11c and the lectin SiglecF, and very low levels of the integrin CD11b.

1.5.2 *Function of Alveolar Macrophages*

Their localization makes AM the first line of defense against inhaled pathogens and therefore crucial for the initiation of the immune response in the lung (Byrne et al., 2015). They additionally contribute to lung homeostasis by maintaining the airways free of pollutants and surfactant and by sustaining an immunosuppressive environment.

Surfactant is a lipoprotein complex that coats the inner surface of alveoli and prevents them from collapsing. It is kept at homeostatic levels through a cycle of constant production by type II alveolar epithelial cells and constant re-

moval by alveolar macrophages (Wright and Dobbs, 1991). Absence of alveolar macrophages results in a condition known as pulmonary alveolar proteinosis (PAP), which is characterized by the accumulation of surfactant in the lung alveoli and consequent respiratory problems. Additionally, PAP patients can present a higher susceptibility to opportunistic lung infections (Trapnell et al., 2003).

Alveolar macrophages also contribute to the process known as airway tolerance, which refers to the maintenance of an immunosuppressive environment that restrict the initiation of immune responses against harmless antigens. Mechanisms that prevent inflammation are crucial in locations that are constantly exposed to external antigens, such as the lung alveoli. Alveolar macrophages show therefore attenuated immune functions in the steady state: in line with the low levels of expression of the phagocytic receptor CD11b, they have a decreased phagocytic activity when compared to other macrophage populations (Holt, 1978) and can poorly present antigens to T cells (Toews et al., 1984; Lipscomb et al., 1986; Lyons et al., 1986). Additionally, they secrete immunosuppressive mediators such as TGF-β and retinal dehydrogenases 1 and 2, that in turn lead to the development of regulatory T cells and the establishment of immune tolerance (Soroosh et al., 2013). Finally, an elegant recent study presented alveolar macrophages as sessile cells that intercommunicate from one alveolus to the other via channels in the lung epithelium. This communication results in periodic waves of calcium release that drive an immunosuppressive environment (Westphalen et al., 2014). Consistent with all these observations, depletion of alveolar macrophages leads to over-inflammatory reactions to harmless particles in the inhaled air (Thepen et al., 1989).

1.5.3 *Development of Alveolar Macrophages*

During embryonic development, several waves of macrophages of different origins populate the lung and contribute to its different resident macrophage populations. Alveolar macrophages, however, arise in the alveoli only during the first week after birth from monocytes that colonize the lung around birth (Guilliams et al., 2013; Tan and Krasnow, 2016).

The establishment and maintenance of alveolar macrophages is GM-CSF dependent (Guilliams et al., 2013; Schneider et al., 2014). In concordance with this, alveolar macrophages are almost absent from mice without functional GM-CSF signaling (Dranoff et al., 1994; Robb et al., 1995; Nishinakamura et al., 1995), leading to the development of pulmonary alveolar proteinosis (PAP). Human alveolar macrophages are also GM-CSF dependent, since natural mutations that affect GM-CSF signaling (Suzuki et al., 2008; Martinez-Moczygemba et al., 2008; Suzuki et al., 2010; Tanaka et al., 2011) or the development of autoantibodies against GM-CSF (Kitamura et al., 1999) lead to a PAP phenotype

in humans as well. It has been shown that the adoptive transfer of macrophages or their precursors with functional GM-CSF signaling (Happle et al., 2014; Suzuki et al., 2014) rescues the PAP phenotype in mice lacking the GM-CSF receptor.

GM-CSF signaling is required to establish alveolar macrophage identity. Particularly, it drives the expression of the transcription factor PU.1 (Shibata et al., 2001) and of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ). PPAR-γ launches an alveolar macrophage-specific gene expression signature that establishes alveolar macrophage identity in the fetal monocytes that colonize the alveoli around birth (Schneider et al., 2014).

1.5.4 *Self-Renewal of Alveolar Macrophages*

In steady state, alveolar macrophages maintain themselves through local self-renewal with minimal contribution from bone marrow-derived monocytes (Janssen et al., 2011; Guilliams et al., 2013; Yona et al., 2013; Hashimoto et al., 2013). This notion can be extended to human alveolar macrophages, since two recent studies showed that donor alveolar macrophages persist in lung transplant recipients at least up to 3,5 years post-transplant (Nayak et al., 2016; Eguíluz-Gracia et al., 2016).

When alveolar macrophages are depleted experimentally through irradiation or by using clodronate-loaded lyposomes, HSC-derived monocytes –rather than local self-renewal- contribute to repopulate the alveolar macrophage niche (Guilliams et al., 2013; Hashimoto et al., 2013; Gibbings et al., 2015). However, when lethally irradiated mice are reconstituted with bone marrow cells that are incapable of colonizing the alveolar niche (for example due to GM-CSFR deficiency), the alveolar macrophage population is replenished by local proliferation of the few alveolar macrophages that survived the irradiation (Hashimoto et al., 2013). Thus, alveolar macrophages retain the capacity to self-renew even after a genotoxic insult. Alveolar macrophage self-renewal utilizes the same mechanisms as MafDKO self-renewal. This is derived from the fact that alveolar macrophages express naturally low levels of MafB and cMaf in the steady state (Gautier et al., 2012; Soucie et al., 2016). Therefore, as MafDKO macrophages, they activate a network of self-renewal genes that drives their proliferation (Soucie et al., 2016). Importantly, alveolar macrophages can be maintain long-term in culture in the presence of GM-CSF, without loosing their identity or the capacity to self-renew (Soucie et al., 2016).

2

LIMITS TO CELLULAR LIFESPAN

2.1 CARREL VERSUS HAYFLICK

At the beginning of the 20th century, the French surgeon Alexis Carrel defended the idea that untransformed cells in culture are immortal, given the right culture conditions. He believed that a limited cellular lifespan was the consequence of metazoan organization, and that cell death was the result of adverse external conditions, rather than an intrinsic characteristic of cells. This hypothesis was based on studies done with chicken embryonic heart fibroblasts, which he claimed to have expanded in culture for 34 years (Carrel, 1912; Ebeling, 1922; Witkowski, 1980). After several unsuccessful trials to reproduce his work in other laboratories, it was generally assumed that primary cells are actually not immortal in culture, and that Carrel's observations were most probably based on a technical artifact. In this matter, a possible explanation was that his team, deliberately or not, added regularly chick embryo extract to the cultures to sustain their growth. Such extract might not only have contained growth factors and nutrients, but also fresh cells that periodically regenerated the cultures. Alternative theories proposed that the cells were accidentally immortalized by a viral infection (Witkowski, 1980, 1985).

The debate was settled when Leonard Hayflick and colleagues published a detailed report on how their own cultures of human fibroblasts actually had a limited replicative capacity in culture. He described how the cells were capable of only a fixed number of doubling times, after which they would simply stop dividing and eventually die (Hayflick and Moorhead, 1961). Importantly, he later showed that the cellular exhaustion he observed was a cell-intrinsic effect, independent of the culture conditions (Hayflick, 1965). Hayflick expanded his observations to a wide variety of human tissues and interpreted them as aging at the cellular level, or cellular senescence. Still today, the limited lifespan of cells in culture is termed the 'Hayflick limit'. All non-transformed cells, with the exception of embryonic stem cells, are subjected to the Hayflick limit (Miura et al., 2004); (Figure I4).

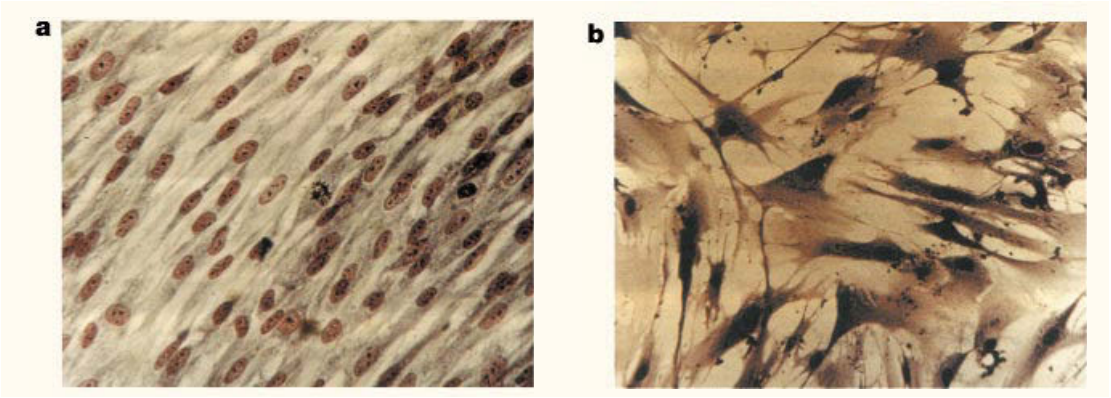

Figure I4: Replicative senescence. Young (a) and old (b) human cells in culture. Source: Shay and Wright (2000).

2.2 HALLMARKS OF AN AGING GENOME

Aging at the cellular level is characterized by a general loss of cellular fitness. Cellular changes that lead to aging are evolutionary conserved. Examples of those changes are: mitochondrial dysfunction, defects in protein homeostasis, deregulated cellular metabolism or genetic instability (Kennedy et al., 2014; López-Otín et al., 2013). Due to the focus of this study, the emphasis of this section is on aging at the genomic level (Figure I5).

2.2.1 *Telomere Exhaustion*

One of the main mechanisms accounting for the replicative senescence known as Hayflick limit is telomere exhaustion (Bodnar et al., 1998). Telomeres are heterochromatin structures composed by tandem DNA repeats and localized at the end of linear chromosomes. They contribute to genome stability by recruiting protein complexes that protect chromosomal ends from degradation and mask them from DNA repair mechanisms. The avoidance of DNA repair at the end of chromosomes is crucial, since these regions would otherwise be handled as DNA breaks and get "repaired", causing chromosome fusion (Palm and de Lange, 2008).

Since conventional DNA polymerases are not able to replicate linear DNA molecules until the end, each cell division shortens telomere sequences. This phenomenon is known as 'end-replication problem' (Olovnikov, 1973). A specialized DNA polymerase, known as telomerase, can compensate for telomere shortening by de novo addition of tandem repeats to the telomeres at the end of each replication cycle (Greider and Blackburn, 1987). Telomerase expression is high during embryonic development and it becomes gradually restricted to the germline and stem cell compartment as the organism matures (Ramirez et al., 1997; Flores et al., 2008), condemning all other adult cell types to a finite

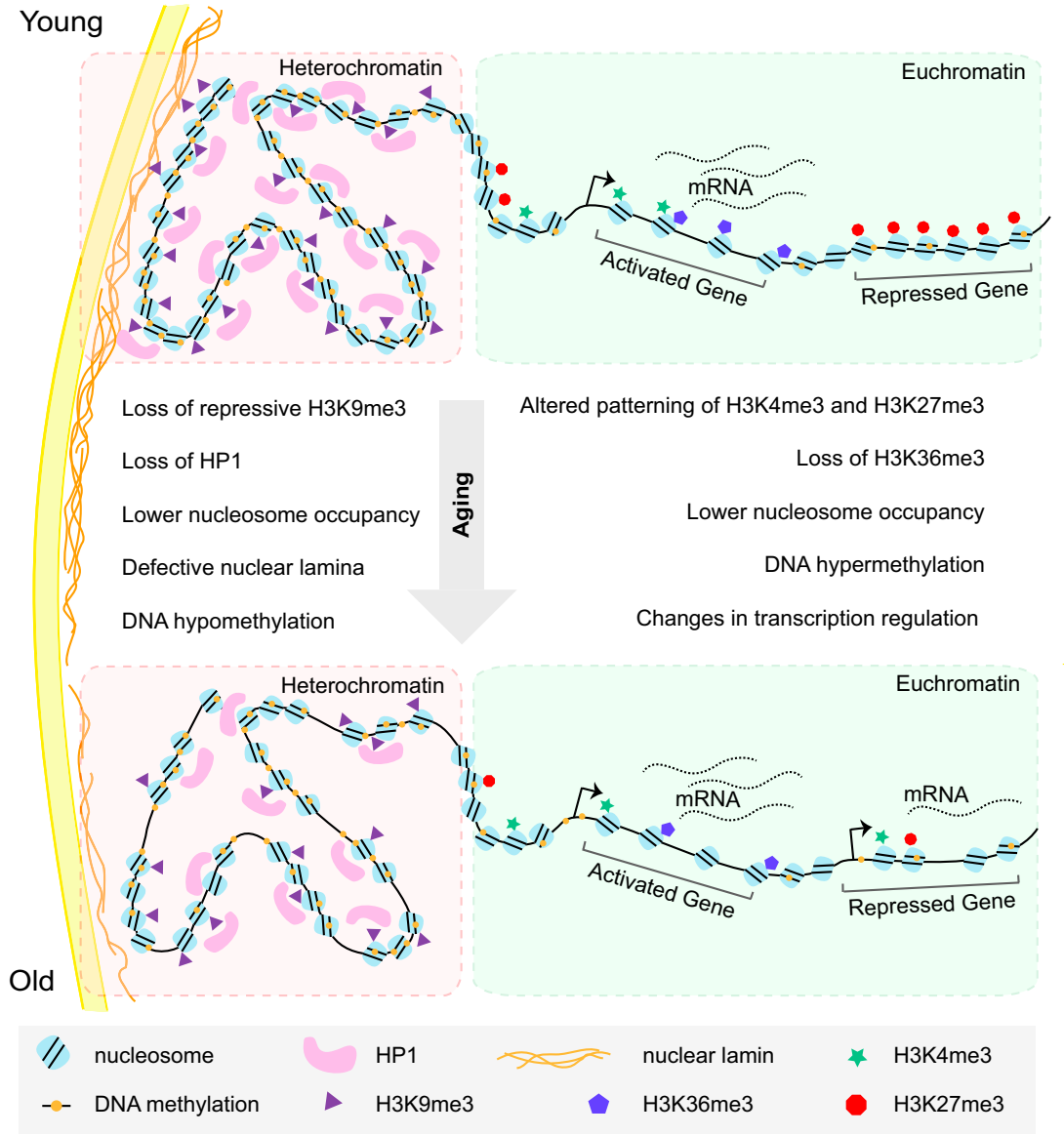

Figure I5: Graphical representation of an aging genome. This illustration presents some of the changes in chromatin architecture that occur during aging. Source: Booth and Brunet (2016).

number of cell divisions. Telomerase expression, however, does not seem sufficient to maintain telomere length, since shortening occurs with age in most tissues (Blasco, 2007).

Progressive telomere exhaustion is therefore a hallmark of senescence at the cellular level and aging at the level of the organism. This notion is confirmed by the observation that mice carrying mutations that shorten telomeres suffer from a shorter lifespan or premature aging-related diseases (Armanios et al., 2009; Herrera et al., 1999; Rudolph et al., 1999), whereas mice with longer telomeres live longer and display an increased general fitness (Tomás-Loba et al., 2008). Consistently, the premature aging phenotype of telomerase-

deficient mice can be reverted by reactivation of the telomerase (Jaskelioff et al., 2010); and ectopic expression of the catalytic subunit of the telomerase is sufficient to immortalize human cells without oncogenic transformation (Bodnar et al., 1998).

The link between telomere shortening and aging is striking also in humans: mutations in telomere maintenance (known as telomere syndromes or telomeropathies) lead to a premature lost of tissue regenerative capacity, reflected in disorders such as pulmonary fibrosis, bone marrow failure or dyskeratosis congenita (Mitchell et al., 1999; Armanios and Blackburn, 2012).

2.2.2 *Epigenetic Remodeling*

Epigenetics are defined as a layer of information above ('epi'-) the DNA sequence of the genome that allows differential gene expression without changing the underlying genetic information. Examples for mechanisms of epigenetic regulation include histone modifications, DNA methylation or nuclear organization. During the chronological aging of an organism, cells undergo important changes in their epigenetic landscape. The relevance of those changes for the induction of cellular senescence is indicated by the fact that interfering with chromatin regulatory complexes has an influence on lifespan (Booth and Brunet, 2016).

DNA Methylation is the covalent addition of methyl groups to DNA bases by methyltransferases. It preferentially occurs at CpG (cytosine followed by guanine) sites in the genome and it is mostly linked to long-term transcriptional silencing (Jones, 2012). DNA methylation is altered in cultured senescent cells when compared to cultured cycling cells (Cruickshanks et al., 2013). Aging cells exhibit a global decrease in DNA methylation in gene-poor regions, such as satellite DNA or lamin-associated domains (LADs) (Cruickshanks et al., 2013; Wilson and Jones, 1983). This leads to genetic instability, since satellite regions are rich in repeated sequences that become active upon loss of DNA methylation and functional LADs are required to anchor chromosomes to the nuclear lamina and contribute to their structure. Additionally, senescent cells present focal hypermethylation at CpG islands near promoters, which is rare under physiological conditions (Cruickshanks et al., 2013; Maegawa et al., 2010) and thus promotes aberrant silencing of genes. Polycomb-targets represent an example of genes silenced by this mechanism during aging (Teschendorff et al., 2010; Beerman et al., 2013).

DNA methylation patterns (termed DNA methylation age) can be used as a very accurate biomarker of chronological and biological age in humans. On the basis of the methylation status of defined CpG islands, several computational models have been able to predict the biological age of human individuals,

human tissues and different cell types (Hannum et al., 2013; Horvath, 2013; Weidner et al., 2014). Some interesting statements derived from calculations by those models are that cell passaging increases DNA methylation age, that embryonic and induced pluripotent stem cells have a DNA methylation age close to zero and that sperm cells are biologically younger than somatic cells from the same donor (Horvath, 2013).

Another aging-induced epigenetic change is an extensive remodeling of the chromatin structure. Changes in histone homeostasis are suggested to contribute to this process since reduction of histone biosynthesis is observed in yeast, cultured mammalian cells and mouse muscle stem cells during aging (Feser et al., 2010; O'Sullivan et al., 2010; Liu et al., 2013). Conversely, elevated expression of histones is enough to extend the life span of yeast (Feser et al., 2010). The pattern of histone modifications along the genome changes during aging as well. As an example, the distributions of H3K27me3 (a repressive histone mark) and H3K4me3 (a mark associated to active transcription) are modified with chronological age in mouse hematopoietic stem cells in vivo (Sun et al., 2014) as well as with replicative age in human lung fibroblasts in vitro (Shah et al., 2013). Another histone mark, H3K36me3, associated with transcriptional elongation and splicing has been related to aging as well. In yeast, its loss results in a shorter lifespan (Sen et al., 2015), whereas deletion of the H3K36me3 demethylase prolongs the lifespan of yeast and C.elegans (Ni et al., 2012; Sen et al., 2015).

Finally, aging also causes a general loss of heterochromatin, or transcriptionally inactive and inaccessible chromatin. In animals, heterochromatin is characterized by H3K9me3 marks and binding of the heterochromatin protein 1 (HP1) (Oberdoerffer and Sinclair, 2007). Accordingly, it has been shown for humans, worms and flies that HP1 and H3K9me3 levels decrease during normal aging (Larson et al., 2012; Ni et al., 2012; Scaffidi and Misteli, 2006; Wood et al., 2010).A global loss of heterochromatin can lead to aberrant gene expression profiles (Tsurumi and Li, 2014).

2.2.3 *Increased Activity of Transposable Elements*

Transposable elements (TEs) are mobile DNA sequences that have the ability to move across the genome. Over the course of evolution, TEs have increasingly colonized eukaryotic genomes in the form of interspersed repeats, to an extent that over 40% of the human genome consists of mobile DNA (Lander et al., 2001).

According to the mechanism they use to propagate, TEs can be divided in two groups: DNA transposons and retrotransposons. DNA transposons encode for an enzyme called transposase. Once synthetized, it binds inverted

repeat sequences at both ends of the transposon, cuts the DNA at those ends and integrates the obtained transposon it into a new genomic locus. DNA transposons are therefore said to mobilize through a 'cut-and-paste' mechanism. For reasons that are not fully understood, DNA transposons are no longer active in human genomes (Pace and Feschotte, 2007). Retrotransposons, on the other hand, function through an RNA-intermediate, which is reverse-transcribed and the corresponding cDNA is subsequently integrated into a new genomic locus. They thus follow a 'copy-and paste' mechanism. Retrotransposition can be further subdivided in two groups: retrotransposons with long terminal repeats (LTR retrotransposons) and without LTR (non-LTR retrotransposons). The latter ones are the most abundant TEs in mammals and are represented by the long and short interspersed nuclear elements (LINEs und SINEs, respectively), In humans, the LINE-1 subfamily dominates transposition, accounting for approximately 17% of the human genome (Burns, 2017).

As mentioned above, aging-induced epigenetic changes can promote TE activity. The retrotransposons Ty1 (Maxwell et al., 2011) and Cer1 (Dennis et al., 2012) have been shown to increase in aging yeast and worms, respectively. In flies, studies using a reporter for de novo TE integration have shown that the gypsy family of LTR retrotransposons increases with age in the brain (Li et al 2013, NN). Similarly, an age-dependent increase of TEs has been reported in the fat body of Drosophila (Wood et al., 2016). Interestingly, depletion of Ago2, an RNA-binding protein that controls TE in fly somatic tissues (Czech and Hannon, 2010), induces an increased transposition in young flies, an impairment of memory with age and a shortening of lifespan (Li et al. 2013, NN), suggesting that TE activity contributes to age-related decline in cellular function. In mice, it has been shown that LINE-1 expression increases with age in the brain. Similarly, cultures of senescent human fibloblasts show a higher number of LINE-1 transcripts than younger controls (Van Meter et al., 2014). Similarly, satellite elements are de-repressed during aging in the mouse liver (De Cecco et al., 2013).

2.2.4 *Accumulation of DNA Damage*

Exogenous agents, such as chemicals or radiation, and endogenous hazards, such as reactive oxygen species, TE activity or errors during DNA replication lead to DNA damage. To ensure cellular fitness, DNA repair mechanisms are constantly active, ready to recognize and correct DNA damage as it occurs (Jackson and Bartek, 2009).

With aging, DNA repair mechanisms become progressively less efficient, causing accumulation of DNA damage over time, consequent genomic instability and eventual cellular senescence (Vyjayanti and Rao, 2006; Sedelnikova et al., 2008). Accordingly, accumulation of double strand breaks with age is ob-

served in normal human cell strains and different mouse organs (Sedelnikova et al., 2004; Singh et al., 2001). Along the same lines, several human disorders of premature aging are a consequence of increased DNA damage, such as the Werner syndrome and the Bloom syndrome (Burtner and Kennedy, 2010).

Experimental evidence for DNA damage-induced aging or senescence reinforces this concept. Mouse embryonic fibroblasts deficient for H2AX, a variant of histone H2A that becomes phosphorylated upon DNA damage to initiate the repair response, exhibit premature onset of senescence in vitro (Celeste et al., 2002). Similarly, mice with mutations in DNA repair proteins exhibit signs of accelerated aging in different tissues or at the level of the organism (Lombard et al., 2005).Importantly, a direct causal link between DNA damage and aging has been indicated by the experimental induction of double strand breaks in mice. Upon systemic administration of a sequence-specific inducible endonuclease and subsequent induction of the enzyme, treated mice display signs of aging in several tissues, such as the liver or the thymus (White et al., 2015; Pinto et al., 2017). Similarly, overexpression of BubR1, a cell cycle checkpoint protein that ensures the correct segregation of chromosomes, extends the lifespan of mice and delays aging-related decline in various tissues (Baker et al., 2012).

3

GATEWAYS TO A TIMELESS EXISTENCE

Intensive research has uncovered various evolutionary conserved mechanisms that counteract age-related damage on the cellular level and therefore positively influence lifespan on the organismal level. Identified mechanisms include signaling pathways such as the insulin/insulin-like growth factor 1 (IGF-1) pathway or the mammalian target of rapamycin (mTOR) pathway, but also broader regulators, such as sirtuins (Kenyon, 2010; López-Otín et al., 2013). The idea of PIWI proteins as anti-aging factors is only starting to emerge. However, based on a careful interpretation of the available data on their function, I also would like to consider them as lifespan guardians. I will present the current state of knowledge about PIWI proteins with respect to a role in lifespan control and will further discuss its implications in the relation to my own data in the discussion.

3.1 SIRTUINS

Sirtuins are enzymes that catalyze post-transcriptional protein modifications of a wide range of proteins, predominantly deacetylation of histones (Haigis and Guarente, 2006). They are defined by their dependency on nicotinamide adenine dinucleotide (NAD+) as an enzymatic cofactor. Due to this dependency, they act as metabolic sensors and regulators of the energy balance in a cell (Bordone and Guarente, 2005). As a family, one of their most remarkable characteristics is their highly conserved role in the regulation of lifespan and longevity.

The yeast silent information regulator 2 (Sir2) is the founder of the sirtuin family. Its overexpression extends the replicative lifespan of yeast, whereas its inactivation shortens it (Kaeberlein et al., 1999)(Kaeberlein 1999). Sir2 controls yeast lifespan by diverse mechanisms, such as protecting the cells from self-replicating extrachromosomal rDNA circles (Sinclair and Guarente, 1997) or regulating cell polarity to ensure that daughter cells receive only undamaged proteins and mitochondria (Liu et al., 2010; Higuchi et al., 2013). Growing

evidence suggests that overexpression of Sir2 homologs in worms and flies also causes a lifespan extension. However, the magnitude of this effect is under debate (Dang, 2014).

Observations on lifespan regulation by sirtuins have also been extended to mammals. The mammalian sirtuin family comprises seven members (SIRT1-7) found in different cellular compartments (Haigis and Guarente, 2006). Similar to observations in invertebrates, SIRT6 overexpression extends the lifespan of male mice (Kanfi et al., 2012). Conversely, mutant mice deficient for SIRT6 exhibit premature aging-associated pathologies (Mostoslavsky et al., 2006). Along the same lines, brain-specific overexpression of SIRT1 induces lifespan extension and delayed aging in mice (Satoh et al., 2013). Interestingly, an ubiquitous overexpression of SIRT1 fails to prolong lifespan but results in an improvement of health span, reflected by an improved glucose homeostasis, improved wound healing, improved neuromuscular function, enhanced cancer protection and delayed bone loss (Herranz et al., 2010). The mitochondria-located SIRT3 has also been involved in longevity, since it mediates the lifespan extension effects of calorie restriction during age-related hearing loss (Someya et al., 2010). Additionally, SIRT3 overexpression has been reported to improve the regenerative capacity of aged hematopoietic stem cells (Brown et al., 2013). Finally, SIRT1 has also been implicated in the maintenance of muscle fitness in young mice. In old mice, SIRT1 and intracellular NAD+ are reduced, resulting in reduced muscle performance (Mohamed et al., 2014).

Because sirtuins can bind and modify a wide range of proteins, the mechanisms by which they regulate lifespan are very diverse. The output of their actions counteracts cellular stress at different levels to ensure homeostasis. SIRT1 maintains DNA stability by the repression of repetitive DNA sequences in steady state and by the induction of DNA repair mechanisms upon genotoxic insults (Oberdoerffer et al., 2008). It can deacetylate the transcription factor heat shock factor 1 (HSF-1), leading to the activation of heat shock proteins such as Hsp70 and thus protecting the cells against protein-mediated stress (Westerheide et al., 2009). Similarly, SIRT6 controls the retrotransposition of LINE1 elements (Van Meter et al., 2014) and promotes DNA repair (Mostoslavsky et al., 2006). SIRT6 can also prevent cellular senescence through histone H3K9 deacetylation. This been shown to prevent telomere dysfunction (Michishita et al., 2008), mitigate NF-κB-dependent signaling (Kawahara et al., 2009) and regulate glucose homeostasis (Zhong et al., 2010), all of which are processes that counteract aging-induced stress.

3.2 THE PIWI/PIRNA PATHWAY

3.2.1 *PIWI Proteins*

PIWI proteins are a family of evolutionary conserved genes that are specifically required for stem cell self-renewal. Their main characterized function is to prevent the mobilization of transposable elements (TEs) in the genome and thus ensure genomic stability. They belong to the Argonaute family of RNA-binding proteins and function in cooperation with a class of small non-coding RNAs known as PIWI-interacting RNAs (piRNAs). Bound piRNAs allow PIWI proteins to identify their targets through base-pairing interactions. Targets of a given PIWI-piRNA complex are mostly silenced, either at the post-transcriptional level through RNA interference, or at the transcriptional level through local epigenetic modifications.

PIWI proteins contain four protein domains: an N-terminal domain, a PIWI-ARGONAUTE-ZWILLE (PAZ) domain, a middle (MID) domain and a PIWI domain (Matsumoto et al., 2016). The N-terminal domain contains dimethhylated arginines that are required for interaction with Tudor proteins, which are important regulators of the PIWI/piRNA pathway (Vagin et al., 2009). The other domains serve the binding, positioning and cleaving of piRNAs: a bound piRNA anchors with its 5'end in the MID-PIWI interface and with his 3'end in the PAZ domain. The PIWI domain has an RNAse H structure capable of cleaving a target RNA if it is fully complementary to the bound small RNA (Matsumoto et al., 2016); (Figure I6).

Across evolution, PIWI proteins are mainly expressed in self-renewing cells and their expression pattern varies among species. In lower organisms with high regenerative capacity, such as the marine flatworm Planaria from the species Schmidtea mediterranea, PIWI proteins are expressed in neoblasts, the totipotent adult stem cells that drive whole-body regeneration (Reddien et al., 2005; Juliano et al., 2011). In Drosophila, they are abundantly found in the male and female germline, but also in somatic tissues such as the somatic stem cells of the gonads or intestinal stem cells in the gut (Juliano et al., 2011; Sousa-Victor et al., 2017; Gonzalez et al., 2015). In mammals, for reasons that are not fully understood, PIWI expression is even more restricted, being almost exclusively found in the male germline (Juliano et al., 2011). A growing number of studies indicate expression of PIWI proteins in somatic mammalian tissues; however, most of them lack robust functional analyses that validate the dignificance of the observed expression. A role for PIWI proteins in mammalian cancer has been proposed as well (Ross et al., 2014).

The founder of the PIWI family, the Drosophila Piwi, was discovered during a P-element induced mutagenesis screening to identify genes that control the

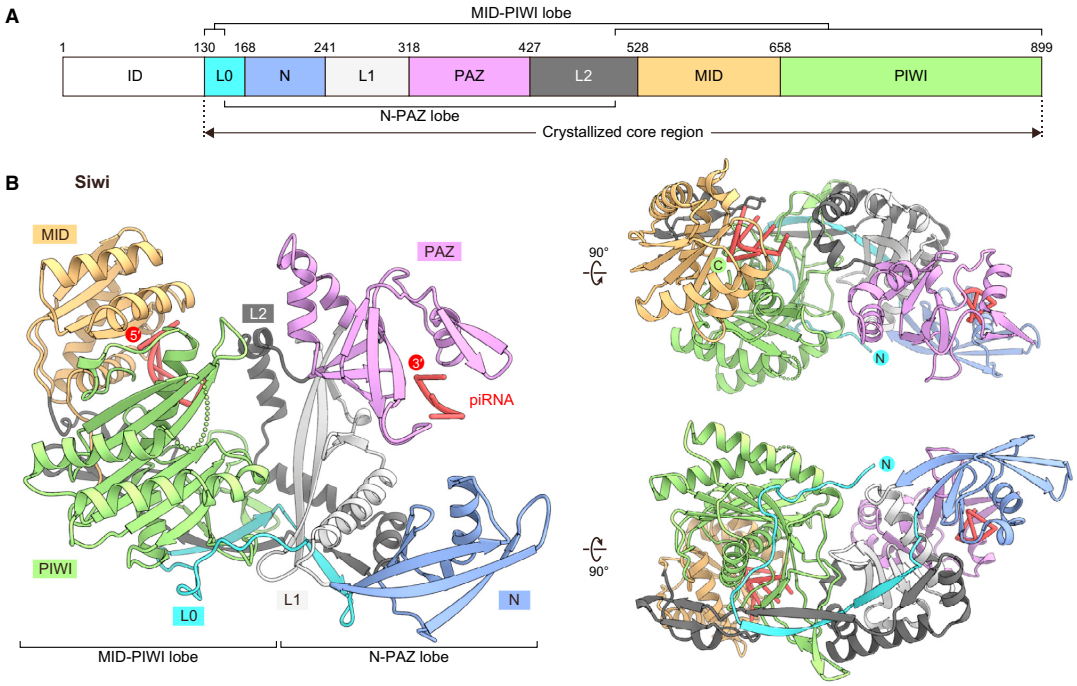

Figure I6: Structure of a PIWI protein bound to a piRNA. This illustration shows the Silkworm PIWI protein Siwi bound to a piRNA. (A) Organization of the Siwi locus. (B) Structure of the SIWI-piRISC complex. Source: Matsumoto et al. (2016).

asymmetric cell divisions of germ stem cells. It gained its name from a striking phenotype caused to the mutant flies: P-element induced wimpy testis, or 'Piwi' (Lin and Spradling, 1997). There are two additional Piwi homologs in Drosophila: Aubergine (Aub) and Argonaute-3 (Ago3). Similarly, the mouse genome encodes three PIWI proteins: Miwi (Piwi-like protein 1, Piwil1), Mili (Piwi-like protein 2, Piwil2) and Miwi2 (Piwi-like protein 4, Piwil4).

For clarity, in all subsequent sections, PIWI refers to the family of proteins, whereas Piwi refers to the Drosophila Piwi protein.

3.2.2 *PIWI-Interacting RNAs*

PIWI-interacting RNAs (piRNAs) are a class of small non-coding RNAs defined by their ability to bind to PIWI proteins. They are single-stranded and comprise 25-32 nucleotides. Their sequences are poorly conserved, even among closely related species (Aravin et al., 2006; Girard et al., 2006; Lau et al., 2006). They are 2'-O-methylated at the 3'end to increase stability (Saito et al., 2007) and arise in their majority from few dedicated loci in gene-poor regions of the genome. These loci, known as piRNA clusters, are abundant in transposable elements (TEs) and can produce piRNAs that map sense or anti-sense to TEs (Brennecke et al., 2007). A smaller fraction of piRNAs can originate from protein-coding genes. Here, the best-characterized source is the 3' untrans-

lated region of some genes (Robine et al., 2009). The most prominent piRNAs of 3'UTR origin in Drosophila arise from the gene coding for the transcription factor Traffic Jam (Saito et al., 2009). Traffic jam is the only Drosophila homolog of the vertebrate transcription factors MafB and cMaf (Li et al., 2003).

piRNAs have been detected in the germline of several species including mice, worms, flies and zebrafish (Vagin et al., 2006; Saito et al., 2006; Brennecke et al., 2007; Aravin et al., 2007, 2008; Houwing et al., 2007), reinforcing the idea that their main function is the maintenance of germline integrity in cooperation with PIWI proteins. As mammalian PIWI proteins, mammalian piRNAs have been described so in the male germline (Aravin et al., 2007; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006).

piRNAs can be produced by two mechanisms: primary and secondary biogenesis. As secondary biogenesis is directly linked with post-transcriptional repression of piRNA targets, it will be discussed in the next section ('3.2.3 PIWI-mediated gene silencing – Post-transcriptional silencing').

piRNAs produced by primary biogenesis are transcribed from genomic regions as single-stranded, long precursor transcripts. They are transported to the cytoplasm and cleaved into smaller piRNAs intermediates. Those intermediates are loaded into PIWI proteins to continue their maturation, which includes trimming and 2'O methylation of the 3' end (Vourekas et al., 2012; Saito et al., 2007; Kawaoka et al., 2011). The length of the obtained piRNA depends on the binding PIWI protein. Each PIWI protein binds to piRNAs of a distinct size range (Brennecke et al., 2007; Aravin et al., 2008). piRNAs derived from primary biogenesis show a strong 5'uracil bias (Brennecke et al., 2007).

piRNAs produced via secondary biogenesis, although derived from a different origin, are loaded into PIWI proteins using the same mechanism as primary piRNAs (Le Thomas et al., 2014).

Depending on the bound PIWI protein, mature PIWI/piRNA complexes have distinct fates. The complex translocates to the nucleus if the bound PIWI protein has usually a nuclear localization. This is the case for Piwi in Drosophila and for Piwil4 in the mouse. By contrast, if the bound PIWI protein normally performs its functions in the cytoplasm, such as Aub or Ago3 in Drosophila, or Piwil1 and Piwil2 in the mouse, the complex remains in the cytoplasm. The localization of the complex also influences the mechanism it uses to silence targets, which can be transcriptional silencing in the nucleus or post-transcriptional silencing in the cytoplasm (Czech and Hannon, 2016).

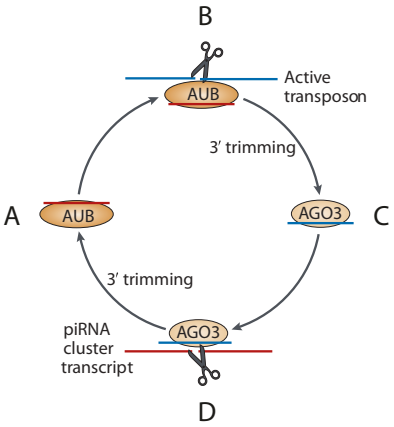

Figure I7: Drosophila ping-pong amplification cycle. (A) The cycle starts when the cytoplasmic PIWI protein Aub is loaded with a primary piRNA that derives from a TE-rich piRNA cluster. This primary piRNA has an antisense orientation with respect to RNA transcripts expressed during TE retro-transposition and therefore directs the Aub/piRNA complex specifically against transcripts derived from active TEs. (B) Once the complex finds a complementary sequence in the RNA transcript pool of the cytoplasm, it binds and cleaves it with the endonuclease activity of the PIWI domain in Aub. This cleavage does not only silence the transposon, but it also represents a source of new RNA fragments that can be further loaded into PIWI proteins. They are termed secondary piRNAs and are sense-oriented to the transposon sequences they derive from. (C) Secondary piRNAs are loaded into another cytoplasmic PIWI molecule, Ago3, where they are trimmed and methylated to complete maturation. (D) Ago3/piRNA complexes in turn recognize and cleave cluster-derived transcripts to generate more antisense piRNAs. The produced antisense secondary piRNAs are then loaded into Aub to restart the cycle. Adapted from Okamura and Lai (2008).

3.2.3 *PIWI-mediated Gene Silencing*

Post-transcriptional Gene Silencing

The PIWI/piRNA pathway mediates post-transcriptional silencing of TEs by a cycling, piRNA-amplifying process known as the 'ping-pong amplification cycle'. This cycle couples secondary piRNA biogenesis with transposon silencing (Brennecke et al., 2007). It takes place in electron-dense cytoplasmic structures known as 'nuage', which are common to all germ cells and compartmentalize all proteins and RNA molecules necessary for piRNA amplification (Pek et al., 2012).

The ping-pong cycle was first characterized in the Drosophila germline and later found to opeate in the mouse male germline as well. It serves as a rapid amplifier of piRNAs directed towards actively jumping retrotransposons (Brennecke et al., 2007; Czech and Hannon, 2016). For clarity, the Drosophila ping-pong is described in the figure legend of Figure I7. The individual contribution of mouse PIWI proteins to the murine ping-pong cycle will be described in section '3.2.5 PIWI proteins in the mouse germline' (Figure I7)

Transcriptional Gene Silencing

The described mechanisms for PIWI-dependent transcriptional silencing of gene expression include the regulation of histone modifications in flies and the establishment of DNA methylation in mice. A role for mouse PIWI proteins in the regulation of histone modifications is suspected but has not been reported. Importantly, Drosophila Piwi has been shown to promote epigenetic activation at specific genomic loci as well (Yin and Lin, 2007; Peng et al., 2016), for example on Polycomb group (PcG) targets through the negative regulation of PcG function (Peng 2016). Since the relevance of these observations for Piwi function and specifically, for the silencing of TEs remains unknown, I will not discuss those studies in more detail.

Depletion of Drosophila Piwi results in loss of heterochromatin and increased RNA polymerase II (Pol II) occupancy at genomic locations of DNA mobile elements, as well as increased levels of nascent TE transcripts (Wang and Elgin, 2011; Sienski et al., 2012; Huang et al., 2013; Le Thomas et al., 2013; Rozhkov et al., 2013). Additionally, it has been shown that Piwi-mediated silencing requires transcription of the target (Pelisson et al., 2007; Sarot et al., 2004). Taking into account these observations, the current model for transcriptional silencing by Drosophila Piwi suggests that mature Piwi/piRNA complexes are recruited to the nucleus, where they scan for nascent RNAs via complementary piRNAs. Following detection, target regions in the genome become transcriptionally silenced through changes in the local chromatin structure. One suggested mechanism for Piwi-dependent chromatin regulation arises from physical interactions between Piwi and chromatin regulators such as heterochromatin protein 1a (HP1a), which in turn attracts histone methyltransferases that subsequently methylate H3K9 to promote the formation of heterochromatin (Brower-Toland et al., 2007; Huang et al., 2013).

DNA methylation is a mechanism of epigenetic control of gene expression absent from flies and present in mammals. Mammalian PIWI proteins show specialized ways to depose DNA methylation marks on TEs. For example the nuclear mouse PIWI protein, Piwil4, gets loaded with piRNAs arising from the cytoplasmic ping-pong cycle (De Fazio et al., 2011)(De Fazio 2011) and translocates to the nucleus to establish CgG methylation on TEs (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). Interestingly, it has also been shown that the murine Piwil2, despite having typically a cytoplasmic localization, can influence DNA methylation by an unknown Piwil4-independent mechanism (Manakov et al., 2015). This recent discovery will be discussed later, as part of the functions of Piwil2 in the mouse germline in the section '3.2.5 PIWI Proteins in the Mouse Germline'.

3.2.4 *PIWI Proteins in the Drosophila Germline*

All Drosophila PIWI proteins, Piwi, Aub and Ago3, are expressed in the male and female germline. As mentioned above, Piwi is a nuclear protein while Aub and Ago3 are predominantly localized to the cytoplasm (Cox et al., 1998; Harris and Macdonald, 2001; Brennecke et al., 2007). Genetic depletion of either Piwi or Aub causes male and female sterility (Lin 1997, Cox 1998, Schmidt 1999), whereas Ago3 is indispensable to female fertility, but only partially necessary for male fertility (Li et al., 2009).

Besides its germline expression, the nuclear Piwi is also expressed in somatic cells of the gonads. Somatic and germline Piwi expression are necessary for the maintenance of female and male germ stem cells (GSCs). Germline Piwi expression is necessary for GSC self-renewal but dispensable for differentiation or commitment of GSC progeny (Lin and Spradling, 1997; Cox et al., 1998; Gonzalez et al., 2015). Consistently, ablation of Piwi in GSCs decreases their division rate and leads to differentiation of GSCs without self-renewing divisions, which results in a gradual loss of the GSC pool (Cox et al., 2000; Gonzalez et al., 2015). In the ovary, somatic Piwi expression in the surrounding GSC niche (specifically in terminal filament cells and cap cells) contributes to germline maintenance (Cox et al., 1998; Szakmary et al., 2005). On the other hand, ablation of Piwi expression in specific somatic cell populations of the gonads: escort cells in the female and cyst cells in the male, results in an accumulation of undifferentiated germline cells, suggesting that somatic Piwi controls GSC differentiation as well (Ma et al., 2014; Gonzalez et al., 2015). Piwi thus supports GSC maintenance and differentiation in a dual manner: in a cell-autonomous manner driving mitosis and by inducing external signals originating in the somatic stem cell niche.

On the other hand, the cytoplasmic AUB and AGO3 proteins are expressed exclusively in the germline of male and female flies. They participate in the silencing of TEs via the ping-pong amplification cycle. Interestingly, the ping-pong cycle can function in the absence of Ago3. However, in the presence of Ago3, antisense piRNA populations directed against actively transcribed TEs are enriched and thus the silencing process is more effective (Li et al., 2009).

3.2.5 *PIWI Proteins in the Mouse Germline*

As mentioned above, there are three homologs of Piwi in the mouse genome: Piwil1, Piwil2 and Piwil4. All of them are non-redundantly required for male spermatogenesis (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007).

Although they collectively repress TEs in the male germline, they have different functions within that process, they have different cellular localizations and they bind to piRNA populations with different characteristics. Consistent with this, they are expressed at different time points during mouse spermatogenesis. To better understand the role of each murine PIWI protein during spermatogenesis, this sub-section starts with a brief description of mouse male germline development, to then discuss PIWI protein function in the mouse germline (Figure I8).

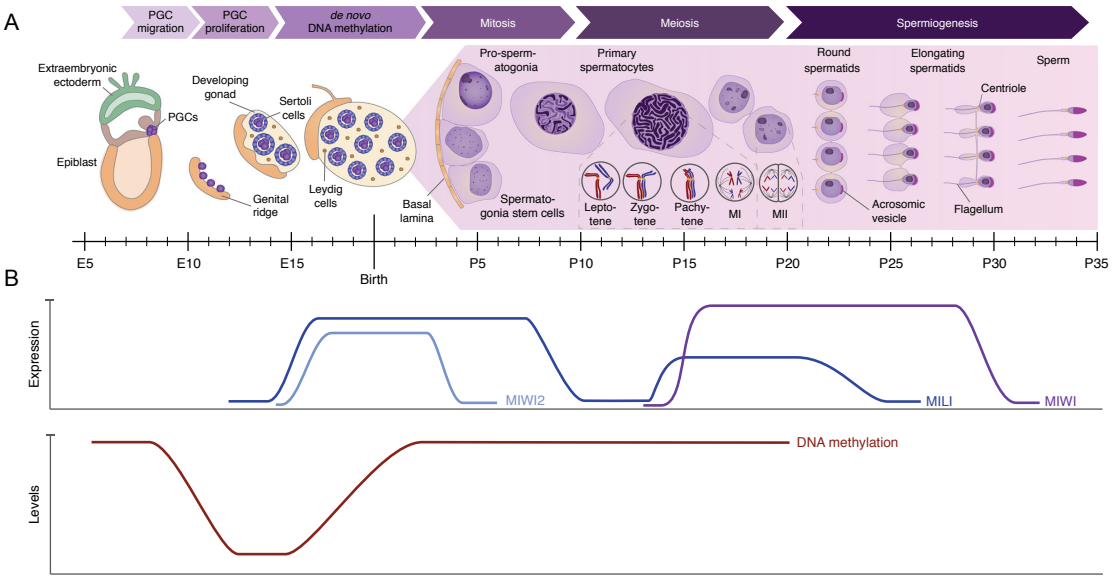

Figure I8: Overview on mouse male spermatogenesis, PIWI expression and DNA methylation. A) Mouse male spermatogenesis from primordial germ cell (PGC) development to sperm with a highlight on the early stages of meiosis (leptotene, zytotene and pachytene). (B) PIWI protein expression and DNA methylation levels of the cells along the process of spermatogenesis. Adapted from Ernst et al. (2017).

Mouse Male Germline Development

Primordial germ cell (PGCs) precursors emerge from the epiblast at embryonic day E7.25. They undergo extensive proliferation to then migrate to the future genital ridges, where the gonads will develop. During this process, they undergo a drastic reprogramming of histone modifications and DNA methylation. This genome-wide remodeling erases parental epigenetic marks to allow the reestablishment of pluripotency and the formation of sex-specific methylation patterns (Feng et al., 2010). In the gonads, PGCs initiate male or female germ cell differentiation according to signals from surrounding somatic cells. Once the male fate is fixed, PGCs differentiate into pro-spermatogonia and undergo cell cycle arrest that lasts until the perinatal period (Rossitto et al., 2015). The establishment of the new DNA methylation patterns takes place during this period of mitotic arrest (Smallwood and Kelsey, 2012).

During the first week after birth, pro-spermatogonia resume proliferation and differentiate into spermatogonial stem cells (SSCs). Spermatogenesis occurs within the seminiferous tubules of the testis. SSCs undergo a series of mitoses whereby daughter cells remain interconnected via intracellular bridges. This leads to the production of primary spermatocytes, which enter the first round of meiosis in a synchronous manner. As cells progress through spermatogenesis, they move from the basal layer of the seminiferous tubules towards the lumen (Bellvé et al., 1977). The second meiotic division gives rise to haploid round spermatids, which are transformed into spermatozoa in the process of spermiogenesis. Spermiogenesis includes drastic morphological changes that include acrosome formation, flagellum development, nuclear condensation and elimination of the cytoplasm. The resulting mature spermatozoa are then released into the lumen of the seminiferous tubules (O'Donnell, 2014).

Piwil1 and Pachytene piRNAs

Piwil1 is a cytoplasmic protein expressed in primary spermatocytes and round spermatids. Germline development in mice deficient for Piwil1 is arrested at the beginning of the round spermatid stage (Kuramochi-Miyagawa et al., 2001; Deng and Lin, 2002). Piwil1 associates with an unusual type of non-classical piRNAs known as pachytene piRNAs. Pachytene piRNAs are very abundant at the pachytene stage of meiosis and remain expressed until the haploid round spermatid stage, to gradually disappear as spermiogenesis advances. Their coordinated increase at the pachytene stage is induced by the transcription factor A-MYB, which also drives the expression of PIWI/piRNA pathway effector genes, such as Piwil1, MitoPld, Mael and Tudor-domain proteins (Li et al. 2013, MC). Pachytene piRNAs get further enriched via ping-pong amplification (Goh et al., 2015), which probably occurs with Piwil2 as cooperation partner of Piwil1, since the expression of both genes overlaps at the pachytene stage of meiosis (Fu and Wang, 2014).

Despite their abundance and punctual expression, the function of pachytene piRNAs function is not fully understood. This relies in part on the fact that most of them do not map to TEs (Deng and Lin, 2002; Aravin et al., 2006; Girard et al., 2006). Several recent studies suggest that pachytene piRNAs target protein-coding mRNAs that are important during meiosis, in order to downregulate them as spermatocytes proceed to round spermatids (Gou et al., 2014; Zhang et al., 2015; Goh et al., 2015).

Piwil2, Piwil4 and Pre-Pachytene piRNAs

Piwil2 is a cytoplasmic protein expressed in the male germline from the PGC stage to the pachytene stage of meiotic primary spermatocytes. Interestingly, at the PGC stage, Piwil2 expression is also found in female gonads (Kuramochi-Miyagawa et al., 2001). spermatogenesis in Piwil2-deficient mice is arrested at the early stages of meiosis, between the zygotene and early pachytene. Despite the expression of Piwil2 in PGCs, PGC develop normally in male and female mice (Kuramochi-Miyagawa et al., 2004).

Piwil4 localizes to the nucleus and is expressed by pro-spermatogonia in the time window when de novo methylation of TE sequences takes place during gametogenesis. Piwil4 expression ceases soon after birth. Piwil4-mutant male mice display a blockage in spermatogenesis in the early prophase of meiosis and a progressive loss of germ cells with age (Carmell et al., 2007).

Piwil2 and Piwil4 associate with classical, pre-pachytene piRNAs enriched in TE-derived sequences. Consequently, mutations of either of both result in de-repression of TEs (Aravin et al., 2007; Carmell et al., 2007; De Fazio et al., 2011; Kuramochi-Miyagawa et al., 2008; Pezic et al., 2014). Piwil2 and Piwil4 coordinate transposon silencing by their participation in the cytoplasmic ping-pong cycle (Aravin et al., 2008). Piwil2 binds piRNAs produced by primary biogenesis (Aravin et al., 2008; Shiromoto et al., 2013) and scans the cytoplasm in the search for target RNA sequences. Complementary sequences are then cleaved by the slicer activity of Piwil2, producing secondary piRNAs that can be loaded into Piwil4. Piwil4/piRNA complexes localize to the nucleus (Aravin et al., 2008) to regulate TEs at the transcriptional level. Piwil4/piRNA complexes resulting from the ping-pong cycle are specially important for the deposition of de novo DNA methylation marks on transposable elements during embryonic reprogramming (Aravin et al., 2008; De Fazio et al., 2011; Kojima-Kita et al., 2018). This is consistent with the fact that mice deficient of either Piwil2 or Piwil4 display de-methylation and de-repression of IAP and LINE1 elements (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008; De Fazio et al., 2011).

Recent evidence suggests that the cooperation between Piwil2 and Piwil4 is dispensable for the silencing of certain families of TEs. Mice deficient for Piwil4 have no change in global levels or composition (e.g. percentage mapping to TEs) of piRNAs (Manakov et al., 2015). Additionally, it has been shown that the ping-pong cycle can occur in absence of Piwil4 (Aravin et al., 2007; Manakov et al., 2015) or in the presence of a Piwil4 mutant that has no endonuclease activity (De Fazio et al., 2011). Piwil2 alone (since Piwil1 is expressed only later) seems thus capable of sustaining an effective piRNA pool that methylates and silences most TE families during embryonic germline reprogramming. Accordingly, Piwil4-deficient mice show methylation defects only in the most active families, such as the LINE-1 subfamilies L1-AT and L1-Gf or the

endogenous retrovirus family IAPEy (Manakov et al., 2015). As mentioned above, the mechanism via which Piwil2 may participate to the silencing of TEs without further PIWI partners is still unknown.

3.2.6 *PIWI Proteins in Somatic Stem Cells of Evolutionary Ancient Organisms*

Despite the strong germline focus in PIWI research, it is important to keep in mind that PIWI proteins are not expressed exclusively in the germline and that PIWI protein expression in somatic cells is also highly evolutionary conserved. Expression of Piwi homologs can be detected in somatic stem cell compartments ranging from the most ancient metazoan organisms, such as sponges, to humans.

Sponges are evolutionary very early-diverging metazoans. They possess two cell types with stem cell-like properties: the totipotent archeocytes and choanocytes, both of which express two Piwi homologs: EfPiwiA and EfPiwiB (Funayama et al., 2010). In animals of the phylum cnidaria (e.g. jellyfish, sea anemones, corals, hydrae), somatic stem cells localized at the base of the tentacles are responsible for the continuous production of stinging cells called nematocytes, and express Piwi homologs as well (Seipel et al., 2004; Denker et al., 2008). Interestingly, in the cnidarian Podocoryna carnea, the PIWI protein Cniwi is not only expressed in adult tentacle bulb cells but also, at low levels, in non-proliferating differentiated cells of the striated muscle. These striated muscle cells are able to trans-differentiate to smooth cells in a process that involves cell cycle re-entry. During trans-differentiation, Cniwi is specifically upregulated during the periods of high proliferation (Seipel et al., 2004).

The genome of Hydra, another cnidarian, encodes for two cytoplasmic PIWI proteins: Hywi and Hyli. Their expression is detected in the germline, in adult somatic stem cells and in progenitor cells. Both PIWI proteins are able to bind to piRNAs in the soma. These piRNAs display hallmarks of ping-pong amplification and map mostly to TEs (Juliano et al., 2014; Lim et al., 2014). Knockdown of Hywi in the somatic epithelial lineage leads to loss of epithelial integrity and eventual death (Juliano et al., 2014). This study provides a functional proof of the importance of PIWI expression in somatic cells of evolutionary ancient organisms.

3.2.7 *PIWI Proteins in Whole Body Regeneration*

Additional studies searching for the functional relevance of PIWI expression in somatic cells of several aquatic organisms uncovered a conserved role for PIWI

proteins in regenerative processes. Two examples of whole body regeneration driven by Piwi homologs are presented below.

Botryllid ascidians, or sea squirts, are colonial tunicates with extraordinary regeneration capacities. They are composed by thousands of genetically identical individuals called zooids. Zooids are embedded in a gelatinous matrix called the tunic and are connected to each other via extracorporeal blood vessels, forming a colony. As a normal part of their life cycle, these colonies undergo weekly cycles of massive apoptosis with subsequent regeneration (Lauzon et al., 1992). This regeneration is driven by circulating somatic stem cells, which migrate to newly grown zooids before the parent zooids undergo apoptosis, efficiently preserving the colony as a whole although single individuals are not preserved (Rinkevich et al., 2013). Natural or experimental induction of regeneration leads to expression of the Piwi homolog BlPiwi in stem cells on the luminal side of the colony vasculature. In response to a regeneration-triggering stimulus, these cells proliferate and migrate though the vasculature, driving the regeneration process. Importantly, inhibition of BlPiwi abolishes colony regeneration (Rinkevich et al., 2010, 2013).

Another well-studied example of whole-body regeneration are Planaria flatworms . Some species of this family can regenerate using totipotent adult stem cells known as neoblasts. Neoblasts are the only mitotic cells in adult planaria and drive regeneration due to their ability to repopulate all somatic and germline lineages in the adult animal (Newmark and Sánchez Alvarado, 2000; Wagner et al., 2011). Interestingly, PIWI have undergone a significant expansion in the genome of planarians, since at least seven Piwi homologs are expressed exclusively in neoblasts (Palakodeti et al., 2008; Friedländer et al., 2009). Three of them (Smedwi1-3) have been functionally characterized.

The knockdown of Smedwi-1 has no effect on regeneration. On the other hand, Smedwi-2 and -3 have been shown to be required for long-term maintenance of neoblasts. Animals deficient for either of these proteins, or for both, display a progressive loss of tissue integrity and eventual death in the steady state. Upon injury, mutant animals are incapable to regenerate, which also leads to death. This dramatic phenotype is due to the incapacity to maintain neoblasts long-term in the absence of Smedwi-2 or -3 (Reddien et al., 2005; Palakodeti et al., 2008).

The presence of piRNAs in planarians has also been reported. Planarian piRNAs are composed by primary and secondary piRNAs and are globally reduced upon knockdown of Smedwi-2 or -3. Although approximately 30% of them map to TEs (Palakodeti et al., 2008; Friedländer et al., 2009), an increase of planarian TEs in the absence of PIWI proteins has not been reported. An additional study suggests that planarian piRNAs may be involved in the silencing of histone mRNA in neoblasts, thus contributing to chromatin organization (Rouhana et al., 2014).

3.2.8 *PIWI Proteins in the Drosophila Soma*

Traffic Jam and the Somatic PIWI/piRNA Pathway

Most Drosophila somatic cells use a simplified version of the PIWI/piRNA pathway that relies on the expression of one PIWI protein only: Piwi. In the absence of the other two PIWI proteins, ping-pong amplification does not take place, resulting in piRNAs that are exclusively produced via primary biogenesis. Somatic piRNAs arise mainly from two sources: the flamenco locus and the Traffic Jam locus (Li et al., 2009; Malone et al., 2009; Saito et al., 2009). The flamenco locus is enriched in TE sequences that are uniformly antisense oriented. Therefore, all piRNAs arising from this locus target transposable elements for their degradation. Interestingly, this locus contains mostly sequences from the gypsy family of LTR transposons (Brennecke et al., 2007). The somatic PIWI/piRNA pathway is therefore a simpler, more straightforward pathway that is directed preferentially against transposable elements belonging to the gypsy family.

Traffic jam (Tj) is the Maf homolog of MafB and cMaf in Drosophila. It is expressed in the male and female somatic gonadal cells that are in direct contact with germline cells during development. Somatic cells in Tj mutant ovaries are unable to envelop germ cells properly, a defect that leads to an early block in germ cell differentiation (Li et al., 2003).

The 3'UTR of Tj has been identified as an efficient producer of piRNAs in the ovarian soma. All piRNAs arising from this locus are sense-oriented, suggesting that Tj transcripts serve as piRNA precursors but do not target their sequence of origin. A potential target transcript of this system is Fasciclin III, a cell adhesion molecule whose de-repression may explain the phenotype observed in Tj mutant ovaries (Saito et al., 2009). Additionally, the TJ protein was shown to activate Piwi expression in female gonadal somatic cells. Accordingly, Tj mutant ovaries lack somatic Piwi expression but conserve Piwi germline expression. This unique mechanism uncovers a dual function for Tj in the regulation of the somatic PIWI/piRNA pathway: the TJ protein induces Piwi expression and the Tj transcript serves as template for piRNA production.

Interestingly, the regulation of the PIWI/piRNA pathway by Tj differs in the testis of Drosophila. Similarly to the ovary, testis deficient for Tj show no Piwi expression in somatic cells. However, germline stem cells from Tj mutant testis express higher levels of Piwi, indicating that Tj negatively controls Piwi expression in the male germline in Drosophila (Saito et al., 2009).

PIWI Proteins in the Somatic Tissue of the Gonads

As mentioned above, Piwi is expressed not only in the germline but also in somatic cells of the male and female gonads in Drosophila. In the ovary, all somatic cells that maintain the GSC niche express Piwi, namely terminal filament cells, cap cells and escort cells (Cox et al., 1998, 2000). In the testis, Piwi is expressed in several somatic support cells, such as hub cells, somatic cyst stem cells and their progeny. Despite the importance of somatic Piwi expression for germline maintenance, somatic cells in the gonads also use Piwi for the transcriptional repression of TEs (Malone et al., 2009; Li et al., 2009). Consistently, down-regulation of Piwi expression in female escort cells results in increased TE activity, increased DNA damage and eventual cell death (Ma et al., 2014). On the same line, testicular cyst stem cells depend on Piwi expression for self-renewal and maintenance (Gonzalez et al., 2015).

PIWI Proteins in the Embryo and in the Adult Brain

All three Drosophila PIWI proteins are expressed in the embryo, in the germline as well as in somatic cells (Mani et al., 2014). Regarding their function, it has been shown that Aub and Ago3 are implicated in the degradation of maternal transcripts during the maternal-to-zygotic transition. This process is piRNA-dependent and involves the recruitment of deadenylation complexes to the target mRNA transcripts to promote their decay (Rouget et al., 2010). Another study has shown that maternal depletion of any of the Drosophila PIWI proteins leads to dramatic mitotic effects in early embryos, originated mainly at a failure to organize chromatin. Mutant embryos display asynchronous nuclear division, aberrant nuclear migration or chromosome fragmentation, and most of them undergo cell cycle arrest before gastrulation (Mani et al., 2014).

Ago3 and Aub have also been associated with the control of TEs in the fly brain. Interestingly, their expression and TE control are not ubiquitous in the brain. By instance, neurons relevant for memory express naturally low levels of Ago3 and Aub and thus show an increased mobilization of TEs. It has been proposed that this might contribute to genomic heterogeneity among memory neurons, possibly providing additional neural diversity (Perrat et al., 2013).

PIWI Proteins and Cellular Lifespan in the Soma

Recent findings indicate that somatic Piwi function might be important for the regulation of lifespan. One study reported expression of Piwi and piRNAs in the adult fat tissue of Drosophila. The piRNAs were mainly TE-derived and were depleted in the absence of Piwi. Consistently, Piwi mutants exhibit increased TE activity and DNA damage in the fat body. This was suggested to

affect fat body function, since mutant flies also exhibit reduced storage of lipid and glycogen and become starvation-sensitive. Although the authors did not show a direct link to fat body function, they also observed that Piwi mutant flies are more sensitive to infections and, intriguingly, display a shorter lifespan than wt animals (Jones et al., 2016).

This last observation is especially interesting in the light of another recent study that describes the role of Piwi during regenerative processes in the gut of Drosophila. This study showed that Piwi expression is induced in intestinal stem cells (ISCs) of Drosophila during periods of high proliferative pressure, such as regeneration. Upon an insult that induces regeneration, ISCs deficient for Piwi exhibit loss of constitutive heterochromatin and increased TE activity, eventually leading to apoptosis. Regeneration thus fails in the absence of Piwi because ISCs are not able to restore the gut epithelium. Interestingly, the study also demonstrated that aging ISCs and challenged Piwi-deficient ISCs share similar features, suggesting that TE activity and DNA damage accumulate over time in ISCs and contribute to their age-dependent decline. Strikingly, Piwi overexpression in intestinal stem and progenitor cells of old flies prevents age-associated dysfunction. Piwi thus appears to be important in the long-term maintenance and function of ISCs and its overexpression seems to be sufficient to prevent age-related stem cell exhaustion (Sousa-Victor et al., 2017).

3.2.9 *PIWI Proteins in the Mammalian Soma*

Evidence for somatic functions of mammalian PIWI proteins remains very scarce. Driven by suggestive observations, two studies have investigated a potential role for PIWI proteins in two mammalian somatic stem cells; the hematopoietic system and induced pluripotent stem cells. In both cases, however, no functional experimental evidence for such a role has been found.

Human hematopoietic stem cells (HSCs) have been reported to express the human PIWI transcript Hiwi. Interestingly, Hiwi expression decreases upon HSC differentiation (Sharma et al., 2001). Expression of Piwil4 has been reported in mouse hematopoietic stem and multipotent progenitor cells, but not in differentiated hematopoietic cells (Nolde et al., 2013). Furthermore, overexpression of Piwil2 in murine bone marrow cells was shown to promote cell proliferation in culture (Chen et al., 2007). All these observations suggested a role for mammalian PIWI proteins in the hematopoietic system. Surprisingly, however, a triple PIWI knockout mouse showed no defects in hematopoiesis in steady state or upon myeloablative injury (Nolde et al., 2013).

Equally, somatic functions of mammalian PIWI proteins have been suggested in induced pluripotent stem (iPS) cells. An interesting study compared the global gene expression profiles of iPS cells derived from fibroblast of hu-

man or non-human primate (NHP) origin and found that Piwil2 was among the top differentially regulated genes. Piwil2 levels were higher in human than in NHP cells, consistent with higher L1 retrotransposition in NHP cells. Strikingly, overexpression of Piwil2 in NHP cells induced a reduction in L1 mobility (Marchetto et al., 2013). However, mouse embryonic fibroblasts deficient for all three mouse PIWI proteins could be efficiently reprogrammed into iPS cells, which were capable of differentiating into all three germ layers in teratoma assays (Cheng et al., 2014), thus leaving an open question about the role PIWI proteins in induced pluripotent stem cells as well.

Part II

EXPERIMENTAL APPROACH

4

RATIONALE

The main function of the PIWI/piRNA pathway is to preserve genome integrity through the repression of transposable elements. An overview of the literature on PIWI proteins reveals that, throughout evolution, they are predominantly active in proliferating cells. In support of the importance of this connection, cells from very different backgrounds in distantly related species, such as cnidarian differentiated muscle cells and Drosophila intestinal stem cells, upregulate PIWI proteins during highly proliferative episodes. Maintenance of genome integrity is of particular importance in cells that undergo self-renewing cell divisions, since their genome is conserved in the population these cells maintain. Accordingly, PIWI proteins are indispensable for the long-term maintenance of various stem cell compartments in the animal kingdom.

It has recently been discovered that macrophages have a stem cell-like capacity to self-renew. Our laboratory has identified several key aspects of this process and shown that macrophage self-renewal relies on the down-regulation of the transcription factors MafB and cMaf. Consistent with this, macrophages deficient for MafB and cMaf, or naturally expressing low levels of these, are capable of long-term self-renewal in vivo and in culture. Mechanistically, self-renewing macrophages activate a gene network that shares multiple functionally important self-renewal genes with embryonic stem cells. MafB represses this network in quiescent macrophages.

Interestingly, the Drosophila Maf homolog Traffic Jam is one of the few known regulators of PIWI function. Additionally, piRNAs derived from the 3'UTR of the Traffic Jam transcript are one of the main sources of piRNAs in somatic tissues of Drosophila.

Based on the observations that

- PIWI proteins have a highly conserved role in stem cell self-renewal
- The Maf homolog Traffic Jam regulates the PIWI/piRNA pathway in Drosophila
- A network of self-renewal genes shared between macrophages and stem cells is regulated by MafB in macrophages

This study aims to answer the following questions:

- Does the PIWI/piRNA pathway play a role in macrophage self-renewal?
- Is MafB involved in the regulation of PIWI proteins during macrophage self-renewal?

5

MATERIALS AND METHODS

5.1 MOLECULAR BIOLOGY

5.1.1 *Nucleic Acid Analysis*

Genomic DNA Extraction (for qPCR – virus efficiency)

For DNA extraction, cells were lysed and genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, 69504) according to manufacturer's instructions.

RNA Extraction

Organs were collected in cold RLT lysis buffer (Qiagen, 79216) and disrupted with a homogenizer. Whole RNA from lysates was extracted using the Qiagen RNAeasy kit (Qiagen, 74104) according to manufacturer's instructions. Sorted cells and cells in culture were collected directly in cold RLT lysis buffer and subjected to the same procedure.

Reverse transcription

Oligo(dT)20 primers (ThermoFisher, 18418020) were used to reverse transcribe RNA using SuperScript^{TM} II Reverse Transcriptase (ThermoFisher, 18064014) according to manufacturer's instructions. When possible, 1µg of RNA was used as starting material.

Real-time qPCR

SYBR® Premix Ex Taq™ II (Takara, RR820Q) was used according to manufacturer's instructions with 10ng of starting complementary DNA (cDNA) or genomic DNA (gDNA). Real-time qPCR was performed in a 7500 Real-Time PCR System (Applied Biosystems). For a list of used primers, please refer to Table A2 in the Appendix.

Tissue processing

Lungs were harvested in wash medium (RPMI, 2% FCS, 1% PS, 30mM HEPES), cut in small pieces and incubated with collagenase II (1mg.mL) and DNase I (0,15mg/mL) for 30min at 37°C. Following incubation, enzymatic activity was stopped by adding PBS with EDTA (10mM). Red blood cells were lysed and the resulting single cell suspensions were filtered and washed.

5.1.2 *Protein Analysis*

Peritoneal macrophages were recovered from the peritoneal cavity by two washes of 10mL each with ice-cold PBS containing 0,5mM EDTA and 5%BSA. The obtained peritoneal wash was subjected to red blood cell lysis and washed.

For microglia preparation, mice were perfused with PBS prior to harvest of the brain. Brains were collected in dissection medium (10mL HBSS, 150µl HEPES, 120µl 45% D-glucose) in sheared to a homogeneous solution. The solution was then filtered, washed and separated by density using a 30/37/70 Percoll gradient. Percoll preparations were centrifuged at 2200rpm for 30min at 4°C. Microglia were harvested from the 37/70 interphase and washed.

Staining for flow cytometry

Single cell suspensions were incubated with mouse FcBlock (BD Biosciences, 553141) and a viability dye (BioLegend, Zombie Fixable) diluted in PBS for 15min at room temperature, protected from the light. Then, cells were washed an incubated with the corresponding antibody-cocktail against surface markers. For intracellular Ki67 staining, cells stained for surface markers were fixed and permeabilized using Cytofix/Cytoperm (BD, 554714) according to manufacturer's instructions. Following fixation and permeabilization, cells were incubated with Ki67 antibody for 1h at 4°C. Following staining, cell suspensions were analyzed on a BD LSRII Flow Cytometer or sorted on a BD FACSAria III using BD FACSDIVA™ software. Data analysis was performed using

FlowJo™ software. For details about used antibodies, please refer to Table A1 in the Appendix.

Proliferation assay by EdU Incorporation

For cell culture, MafDKO macrophages were incubated for 3h with 10uM EdU 37°C under 5%CO_2. Following incubation, cells were detached from culture dishes, stained for surface markers and subjected to detection of incorporated EdU using the Click-iT™ Plus EdU kit (ThermoFisher C10640) according to manufacturer's instructions. For in vivo experiments, EdU was administered to mice intra-peritoneally 4h before tissue harvesting. Harvested tissues were processed to a single cell suspension, stained for flow cytometry and subjected to incorporation of EdU detection using the kit mentioned above.

DNA content assessment by flow cytometry

When needed, FACS staining and assessment of EdU incorporation assessment was combined with DNA content analysis. For this purpose, cells were stained with FxCycle™ Violet Stain (Thermofisher, C10640) after the EdU protocol according to manufacturer's instructions.

Apoptosis analysis by flow cytometry

Assessment of apoptosis was performed using the Caspase3 (active) Staining Kit (Abcam, 65613) according to manufacturer's instructions.

5.2 CELL BIOLOGY

Bone marrow-derived macrophages

Bones from irradiated CD45.1 mice reconstituted with either MafDKO or WT CD45.2 fetal liver were collected and flushed with cold PBS to obtain total bone marrow cells. Red blood cells were lysed and remaining cells were washed. Cell suspensions were incubated with a biotin-coupled antibody against CD45.1 and then with an anti-biotin microbeads according to manufacturer's instructions (Miltenyi Biotec, 130-090-485). CD45.1+ cells were depleted using an AutoMacs Pro Separator. Remaining cells were cultured at 37°C under 5%CO_2 in complete medium (DMEM, 10% heat-inactivated fetal calf serum (FCS), 1% Penicilin/Streptomycin, 1% L-Glutamine, 1% Sodium pyruvate) supplemented with 20% supernatant of M-CSF producing L929 cells. Medium was replaced

partially (50%) every three days until cultures completed 14 days. From 14 days on, cells were considered mature bone marrow-derived macrophages as judged by FACS analysis of expressed surface markers.

Harvest of alveolar macrophages by bronchoalveolar lavage (BAL)

Alveolar macrophages were harvested from the alveolar cavity by several washes with warm BAL wash medium (PBS without Mg2+/Ca2+, 2mM EDTA, 2%FCS). The washes were done by inserting a syringe with a blunt needle directly into the exposed mouse traquea. Each mouse was washed 10 times with 1mL wash medium. Cells were harvested in cold RPMI medium. Following harvesting, red blood cells were lysed and remaining cells were washed.

Culture of MafDKO macrophages

MafDKO macrophages were cultured at 37°C under 5%CO_2 in complete medium (DMEM, 10% heat-inactivated fetal calf serum (FCS), 1% Penicilin/Streptomycin, 1% L-Glutamine, 1% Sodium pyruvate) supplemented with 20% supernatant of M-CSF producing L929 cells. Cells were detached from culture plates using 1xTrypsin for 5min at 37°C under 5%CO_2. When necessary, cells were frozen in heat-inactivated FCS containing 10% of DMSO was used as freezing medium. Cumulative population doublings were calculated as shown elsewhere (Greenwood et al., 2004).

Culture of alveolar macrophages

Alveolar macrophages were cultured in non-treated (bacterial) dishes at 37°C under 5%CO_2 in complete AM medium (RPMI, 10% heat-inactivated fetal calf serum (FCS), 1% Penicilin/Streptomycin, 1% L-Glutamine, 1% Sodium pyruvate) supplemented with 4% supernatant of GM-CSF producing mouse macrophages J558L. Cells were kept confluent at all times. Cells were detached from culture plates using Accutase + mM EGTA. When necessary, cells were frozen in heat-inactivated FCS containing 10% of DMSO was used as freezing medium.

Spin infection of MafDKO macrophages with lentiviral supernatant

MafDKO macrophages were seeded confluently one day before the infection. On the day of the infection, lentiviral supernatant was incubated with 8µg/mL polybren for 1,5h at 37°C under 5%CO_2. Following incubation, lentiviral supernatant was added to the cells are the cells were centrifuged for 2h at 2500rpm

at room temperature. After spin, cells were kept with the lentiviral supernatant for further 30 min at 37°C under 5%CO_2. Cells were selected for 8 days with 4µg/mL puromycin prior to all performed assays.

Colony Formation Assay (CFA)

1000 MafDKO macrophages were plated in MethoCult^{TM} M3231 (Stemcell Technologies, 03231) supplemented with Penicilin/Streptomycin, 100ng/mL recombinant M-CSF and 4µg/mL puromycin. CFAs were maintained at 37°C under 5%CO_2 for 14 days prior to counting the colonies.

10000 alveolar macrophages were plated in MethoCult^{TM} M3231 (Stemcell Technologies, 03231) supplemented with Penicilin/Streptomycin and 100ng/mL recombinant GM-CSF. CFAs were maintained at 37°C under 5%CO_2 for 21 days prior to counting the colonies.

5.3 IN VIVO PROCEDURES

Intratraqueal delivery of antibodies, drugs (clodronate liposomes) or cells

Mice were anesthetized by an intraperitoneal injection of Tribromoethanol (Avertin, 250mg/kg). Once deep sleep was achieved, a syringe with a short gavage needle containing the substance to be delivered was inserted into the traquea through the wide-open mouth of the mouse. The volume for the delivered substance never exceeded 100µL.

Adoptive transfer of alveolar macrophages by intranasal transplantion in newborn mice

Newborn mice in the first week of life were anesthetized using isoflurane. In the meantime, a pipette containing 30000 cells in 7µL cold PBS was prepared. Following the very first movement of the mouse upon recovery, cells were deposed as a single drop on top of one nostril.

5.4 MICE

C57BL/6J WT mice were obtained from JANVIER LABS. MafDKO (and WT control) mice used to generate bone marrow-derived macrophages were obtained by transplanting CD45.2 MafDKO or WT fetal liver into lethally irradi-

ated CD45.1 WT recipients. This strategy is adopted because mice deficient for MafB die at birth. Piwil2-KO mice (Di Giacomo et al., 2013) were kindly provided by Donal O'Carroll. Csf2rβ-KO mice (Robb et al., 1995) were provided by Melanie Greter. Conditional MafB-KO (Matcovitch-Natan et al., 2016) mice were created in our laboratory.

6

RESULTS

6.1 PIWI PROTEINS IN SELF-RENEWING MAFDKO MACROPHAGES

Macrophages with a simultaneous deletion of the myeloid transcription factors MafB and cMaf (MafDKO macrophages) are used in our laboratory as an in vitro system for the study of macrophage self-renewal. As mentioned in the Introduction, MafDKO macrophages (as opposed to WT counterparts) can be maintained and expanded in culture over indefinite passages. In this study, MafDKO macrophages and corresponding WT controls were derived from the bone marrow of mice with a MafDKO or WT reconstituted hematopoietic system. This strategy is adopted because mice with a ubiquitous MafB deficiency die at birth.

6.1.1 *Self-Renewing Macrophages Express a Truncated Form of PIwil2*

As a first step to answer the question whether PIWI proteins play a role in macrophage self-renewal, we tested for expression of the three mouse PIWI family members in self-renewing macrophages (MafDKO) and their counterpart controls (WT). We found by RT-qPCR (Figure 1) and by RNA Sequencing (Figure 1B) that macrophages expressed detectable levels of Piwil2 and very low levels of Piwil4. We did not find Piwil1 expression. Interestingly, Piwil2 and Piwil4 were expressed at higher levels in MafDKO than in WT macrophages. We decided to focus our further studies on Piwil2.

We then assessed the reads mapping along the Piwil2 locus in our RNA Sequencing data and discovered that the Piwil2 mRNA expressed by macrophages was not full-length, but N-terminal truncated. Reads mapping to the Piwil2 locus started approximately at exon 11 and ended at the last exon of the locus (Figure 2A and 2B). Since it had been reported that the Piwil2 gene can be expressed as a set of shorter, mostly N-terminal truncated isoforms implicated in cell proliferation in cancer cells (Ye et al., 2010), we hypothesized

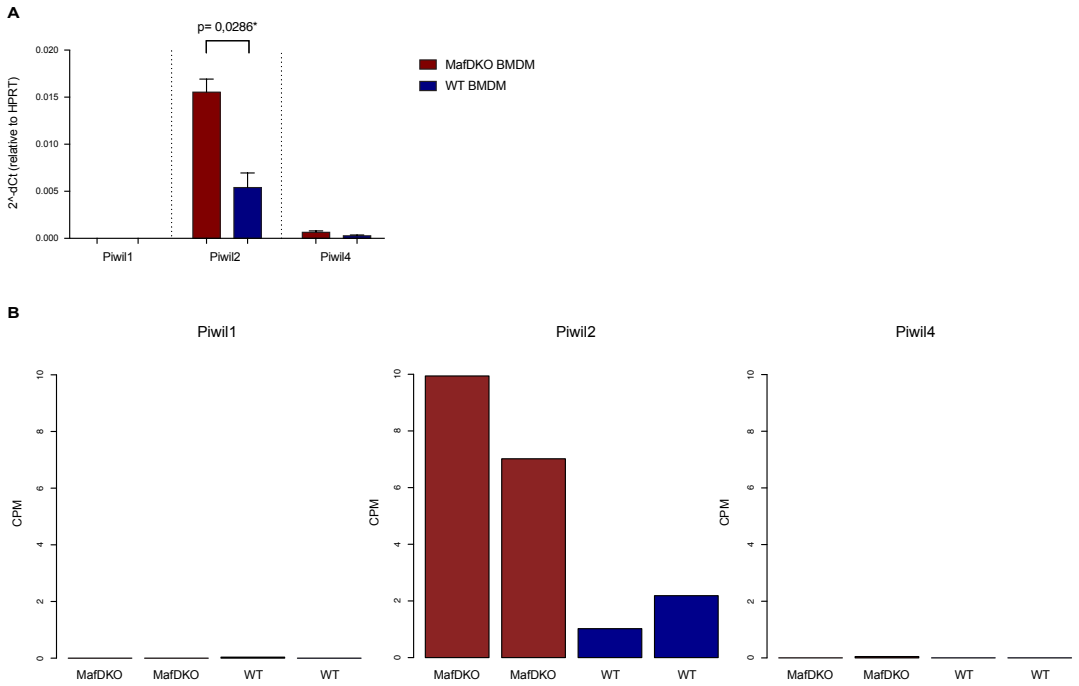

Figure 1: Expression of PIWI family members in macrophages. (A) Expression of the three murine PIWI family members in MafDKO or WT macrophages, analyzed by RT-qPCR. Values are normalized to HPRT. Data are presented as the mean of three independent experiments. Error bars indicate SD. P-value was calculated using the Mann-Whitney Test. (B) Expression of the three murine PIWI family members analyzed by RNA-Sequencing in Maf-DKO or WT macrophages. Number of reads are normalized to sequencing depth and plotted as "count per million" (CPM). The graph shows two independent experiments.

that the shorter isoform of Piwil2 is a functional PIWI protein. We named this isoform 'Piwito'.

Despite several efforts, we could not find a specific antibody against the C-terminal portion of PIWIL2. Therefore, to further validate the expression of Piwito in macrophages, we decided to evaluate the epigenetic status of the Piwil2 locus in MafDKO and WT macrophages. For this, we interrogated data available in our laboratory on chromatin immunoprecipitation coupled with deep sequencing (ChIP-Sequencing) of MafDKO and WT macrophages. ChIP-Sequencing analyses were done using several DNA-binding proteins: to define enhancer and promoter regions in macrophages, we used binding of the transcription factor PU.1, which has been shown to define macrophage- and B cell-specific genomic regions (Natoli, 2010; Ghisletti et al., 2010; Heinz et al., 2010). To analyze genome-wide histone modification signatures, we assessed for the presence of mono- and tri-methylation of histone H3 at Lysine 4 (H3K4m1 and H3K4m3 respectively). Lastly, to gain knowledge about active enhancer regions, we used binding of histone acetyltransferase p300 as well as the histone modification mediated by this enzyme: acetylation of histone 3 lysine 27 (H3k27ac) (Creyghton et al., 2010; Ostuni et al., 2013; Visel et al., 2009).

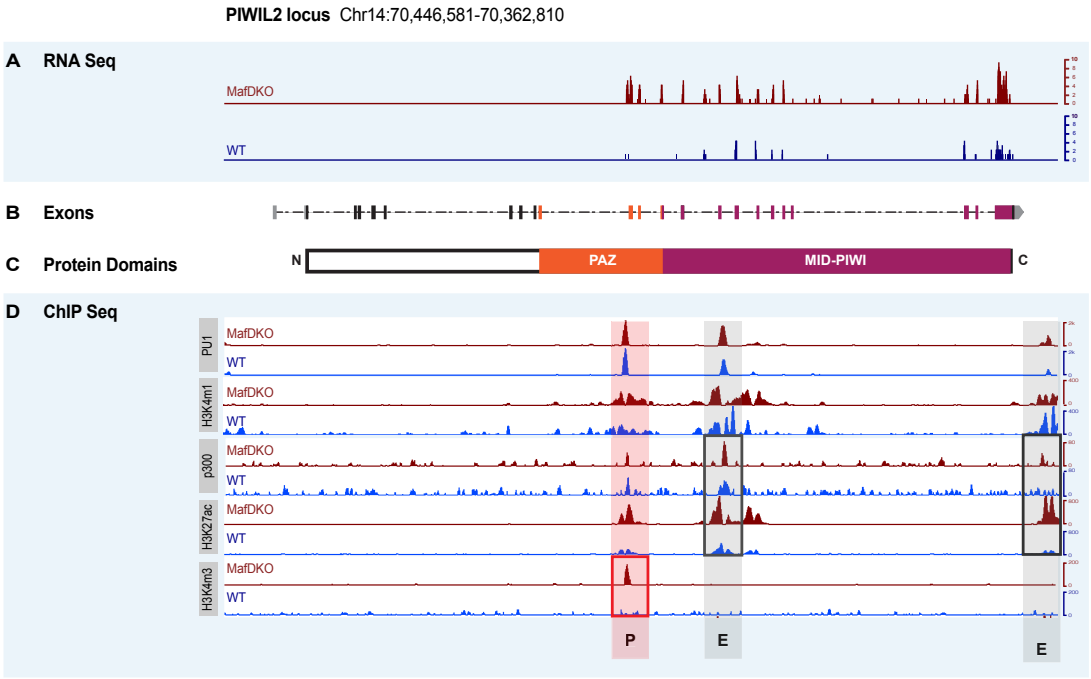

Figure 2: RNA- and ChIP-Sequencing analysis of the Piwil2 locus of macrophages. (A) RNA-Sequencing reads mapping to the genomic region of the Piwil2 locus in MafDKO and WT macrophages. (B) Scheme depicting exon-intron organization along the Piwil2 locus. Tick marks represent exons. Colors of the exons match the protein domains they code for. Grey-colored exons are part of the untranslated region of the locus. (C) Scheme of the translated Piwil2 protein and localization of its protein domains. (D) Genomic region corresponding to the Piwil2 locus in MafDKO or WT macrophages with ChIP-Sequencing tracks as labeled. Regions predicted as an internal promoter (P) or as enhancers (E) are emphasized by red and grey shading respectively. One representative experiment is shown.

Binding of PU.1 revealed three interesting regions along the Piwil2 locus in MafDKO and WT macrophages (Figure 2D). Two of those regions were classified as internal enhancers (Figure 2D, grey shading) as they revealed a typical enhancer signature: H3K4m1+/H3K4m3low/-. Interestingly, these enhancers were active in MafDKO macrophages but not in WT counterparts. This was indicated by stronger signals for p300 and H3k27ac (Figure 2D, grey box).

Actively transcribed promoters (and associated transcription starting sites (TSSs)) can be identified by the chromatin mark H3K4m3. The third PU.1-binding region in the Piwil2 locus of macrophages showed an H3K4m3 mark in MafDKO macrophages but not in WT controls (Figure 2D, red box). Therefore we concluded that this region was the promoter of Piwito and that Piwito was being actively transcribed in MafDKO macrophages.

We were then interested to know which was the first Piwil2 exon being transcribed in macrophages. For this purpose, we designed primers that targeted exon-exon-junctions along the entire length of the Piwil2 mRNA (Figure 3A) and used them to scan the Piwito mRNA in MafDKO and WT macrophages

by RT-qPCR. We found that Piwito mRNA started with exon 11 and ended with Exon 23 (Figure 3B).

Taken together, our results show that macrophages express a shorter isoform of Piwil2, comprising exons 11 to 23 of the full-length Piwil2 mRNA. This short isoform is upregulated in self-renewing macrophages when compared to quiescent macrophages.

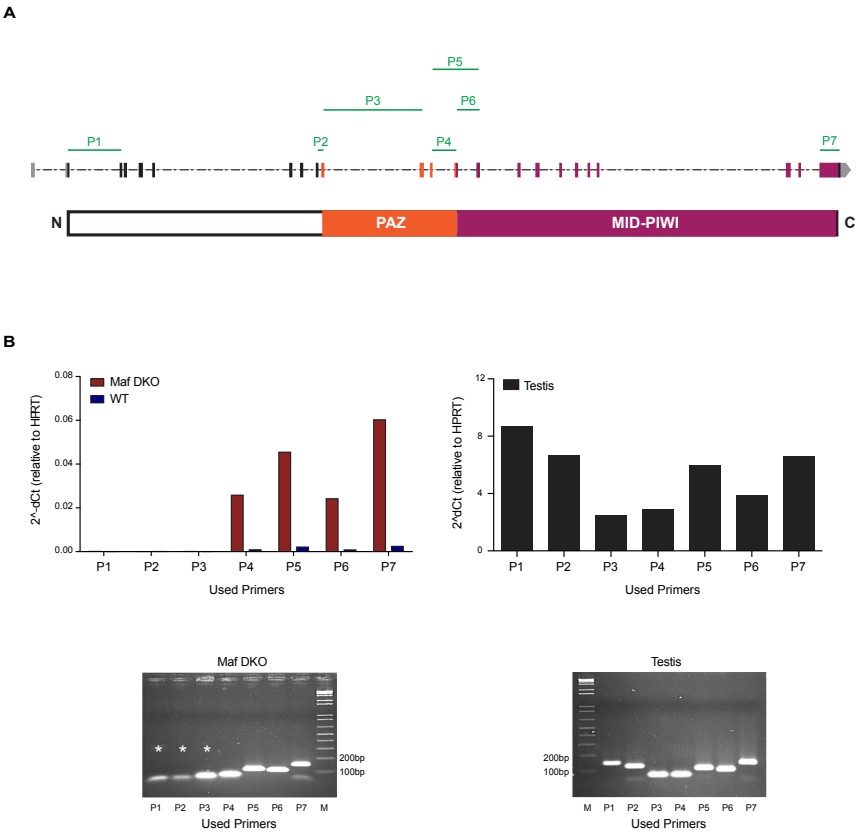

Figure 3: mRNA expression from the Piwil2 locus in macrophages. (A) In green: localization of the primer amplicons used in (B) to scan the complete Piwil2 locus by RT-qPCR. A scheme under the Piwil2 locus shows the translated Piwil2 protein and the localization of its protein domains. (B) Scan of the Piwil2 locus in MafDKO macrophages, WT macrophages and whole testis lysates (positive control) by RT-qPCR. Upper panel: Values represent mRNA expression normalized to HPRT. Lower panel: Obtained PCR products loaded on an agarose gel for visualization. One representative experiment is shown. White asterisks mark lanes with no specific PCR amplicon.

6.1.2 *Piwito Regulates the Self-Renewal of MafDKO Macrophages*

To assess whether Piwito plays a role in the self-renewal of MafDKO macrophages, we first tested if Piwito levels could be influenced by M-CSF, the cytokine that promotes the growth of MafDKO macrophages. Interestingly, we observed that Piwito expression was induced in MafDKO and WT macrophages upon stimulation with M-CSF (Figure 4). As seen in earlier experi-

ments, the overall levels of Piwito were higher in MafDKO macrophages when compared to WT controls. Since M-CSF induces macrophage proliferation, and given the conserved role of Piwil2 in self-renewal, we hypothesized that Piwito plays a role in the self-renewal of macrophages.

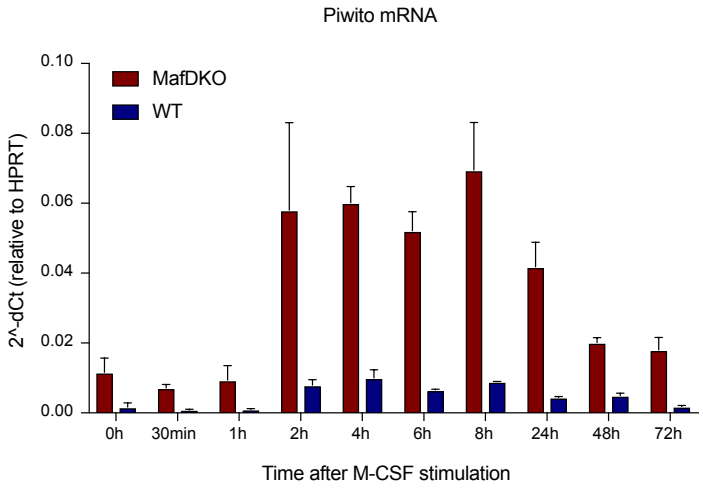

Figure 4: Expression of Piwito mRNA upon M-CSF stimulation Values for Piwito mRNA normalized to HPRT at different time points after M-CSF stimulation (20ng/mL). Data are presented as the mean of three independent experiments. Error bars indicate SD.

We tested this hypothesis by performing functional proliferation studies on MafDKO macrophages in the presence or absence of Piwito. To achieve this, we used lentiviruses to deliver short hairpin RNA (shRNA) probes against Piwito to MafDKO macrophages. We tested four different shRNA probes for infection and knockdown efficiency. Since all our lentiviral vectors carry a puromycin resistance, we assessed infection efficiency by evaluating the presence of puromycin copies in the genomic DNA of infected MafDKO cells by qPCR 48h after infection. The infection efficiency was comparable among all used shRNA vectors, including our empty vector (EV) control (Figure 5A). We then checked the knockdown efficiency of our vectors by looking at Piwito expression by RT-qPCR, also 48h after infection. The most efficient knockdown was achieved by two of our four shRNA vectors: sh3 and sh4 (Figure 5B). We decided to use those two vectors for our functional studies. Following infections and before the functional studies, successfully infected MafDKO macrophages were selected in puromycin-supplemented growth medium.

First, we followed the growth of MafDKO macrophages expressing shRNAs against Piwito (sh3 and sh4) or an EV control in liquid culture over time. As soon as 5 days after infection, cells expressing sh3 or sh4 showed a slower growth when compared to cells carrying an empty vector (Figure 6A). To further assess the proliferation capacity of the cells, we plated MafDKO macrophages infected with sh3, sh4 or EV in a semi-solid medium at single-cell-density and counted the colonies that arise from each condition 14 days after platting (Colony Formation Assay, CFA). We observed a significant reduction

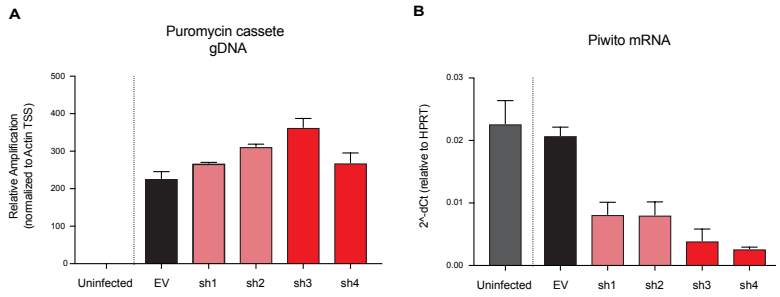

Figure 5: Infection efficiency of lentiviral particles carrying shRNA or controls in MafDKO macrophages. MafDKO macrophages were infected with lentiviral particles carrying different shRNAs or an empty vector (EV) control. gDNA and total RNA were analyzed 48h after infection. (A) qPCR on genomic DNA of infected MafDKO macrophages. Values represent amplification of the puromycin cassette normalized to Actin TSS. (B) RT-qPCR of Piwito-mRNA on MafDKO macrophages. Values are normalized to HPRT. One representative experiment is shown.

in colonies in MafDKO cells upon expression of any of both shRNAs against Piwito (Figure 6B). Finally, we used flow cytometry to gain knowledge about the status of the cell cycle in our cells. To this purpose, we measured DNA content using a DNA dye and tracked DNA synthesis by detecting the incorporation of the nucleoside analog 5-ethynyl-2 deoxyuridine (EdU) into newly synthetized DNA. This assay revealed that, overall, fewer cells were proliferating upon expression of a shRNA against Piwito as compared to the EV control (Figure 6C). More specifically, knockdown of Piwito resulted in an increase of cells in G0/G1 and a linked decrease of cells in the S-Phase, suggesting a cell-cycle arrest at G0/G1 (Figure 6D). Importantly, we also checked for apoptosis using a flow cytometry-based assay that detects levels of active caspase 3 and found no difference between MafDKO cells expressing shRNAs against Piwito or an EV control (Figure 6E).

Our results show by several functional assays that the knockdown of Piwito compromises the self-renewal of MafDKO macrophages. Importantly, the defect does not rely on increased apoptosis of Piwito-deficient cells.

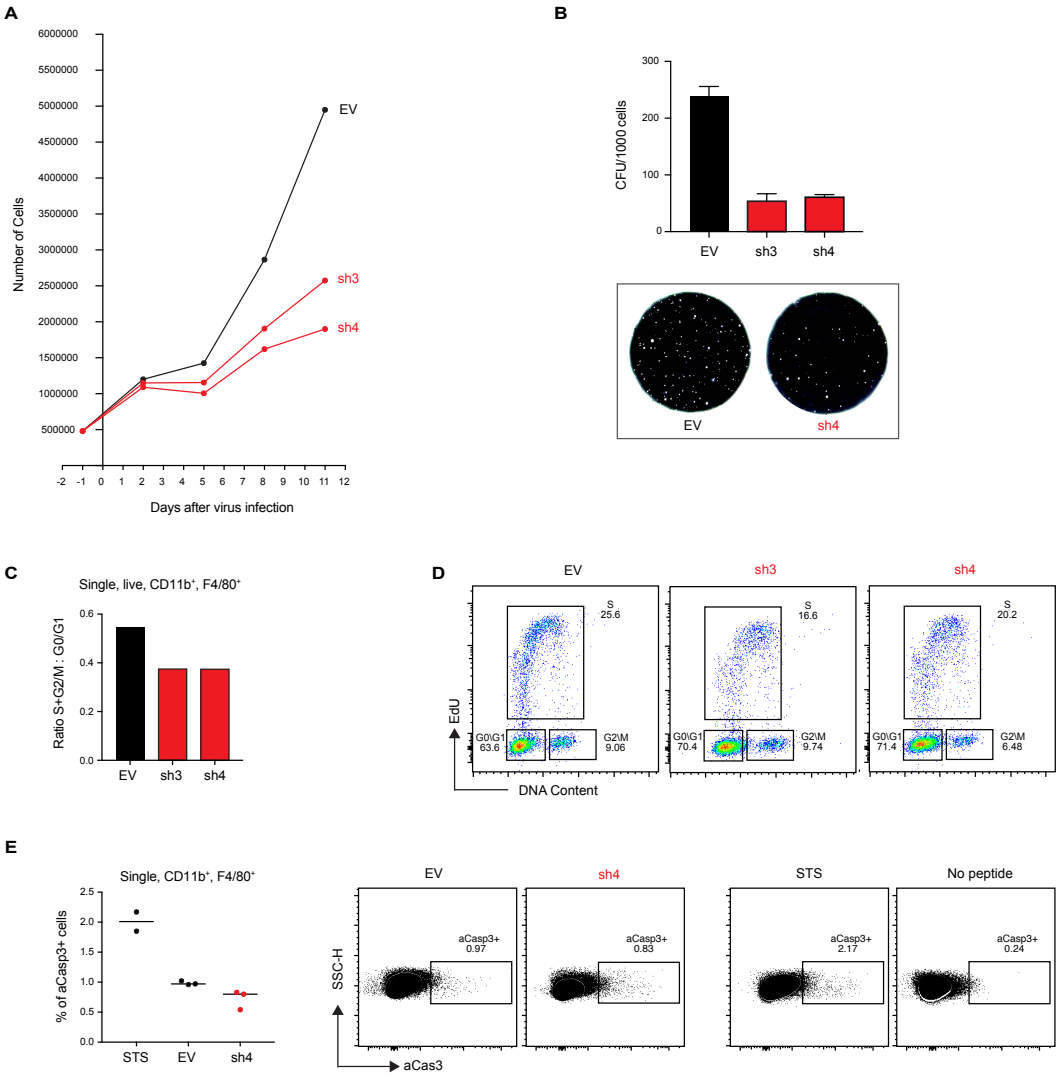

Figure 6: Proliferation Assays on MafDKO macrophages expressing shRNAs against Piwito. MafDKO macrophages were infected with lentiviral vectors carrying shRNAs against Piwito (sh3 and sh4) or a corresponding empty vector (EV) control. Following infection and prior to analysis, cells were selected in Puromycin-containing medium for 8 days. (A) Cell numbers of puromycin-resistant MafDKO macrophages grown in liquid culture for 12 days in the presence of puromycin (B) Colony Formation Assay (CFA) of puromycin-resistant MafDKO macrophages in the presence of recombinant M-CSF and puromycin. Upper graph depicts colony counts per 10000 initially plated cells 14 days after plating. The lower graph shows pictures of representative CFA dishes for sh4- and EV-expressing macrophages. (C-D) Flow cytometry-based cell cycle analysis of puromycin-resistant MafDKO macrophages for the assessment of DNA content and EdU incorporation. (E) Flow cytometry-based assay for the detection of cells expressing active caspase3 in puromycin-selected MafDKO macrophages. Positive control: staurosporine (STS)-treated MafDKO macrophages. For each analysis, one representative experiment is shown.

6.2 PIWI PROTEINS IN ALVEOLAR MACROPHAGES

To investigate whether our observations in MafDKO macrophages are relevant to macrophages with no genetic modifications, we explored the role of Piwito in the self-renewal of alveolar macrophages (AM). AM are the resident macrophage population of the lung alveoli. We focused on this population for two important reasons. First, they have the capacity to self-renew and maintain their population independently from hematopoietic stem cells (Hashimoto et al., 2013; Soucie et al., 2016). Second, AM maintain naturally low levels of MafB and cMaf and share self-renewal mechanisms with MafDKO macrophages (Gautier et al., 2012; Soucie et al., 2016). Additionally, AM offer several practical advantages: they can be easily harvested from the alveolar space as an almost pure population that requires no further enrichment for most applications and, in our laboratory, we are able to establish and maintain long-term cultures of them (Soucie et al., 2016).

6.2.1 *Piwito is Expressed in Alveolar Macrophages*

We isolated RNA from freshly harvested alveolar macrophages and tested by RT-qPCR for expression of all three murine PIWI members. In analogy to MafDKO macrophages, Piwil2 transcripts were expressed in alveolar macrophages, whereas Piwil1 and Piwil4 transcripts were not detectable (Figure 7A). We wondered if alveolar macrophages expressed the full-length Piwil2 or its shorter isoform, Piwito. To answer this question, we scanned the full-length Piwil2 mRNA in AM by RT-qPCR using primer pairs that target exon-exon junctions along the transcript. We observed that the detected mRNA arose from Piwito, and not from the whole length Piwil2 (Figure 7B). This result was further confirmed by RNA-Sequencing (Figure 7C).

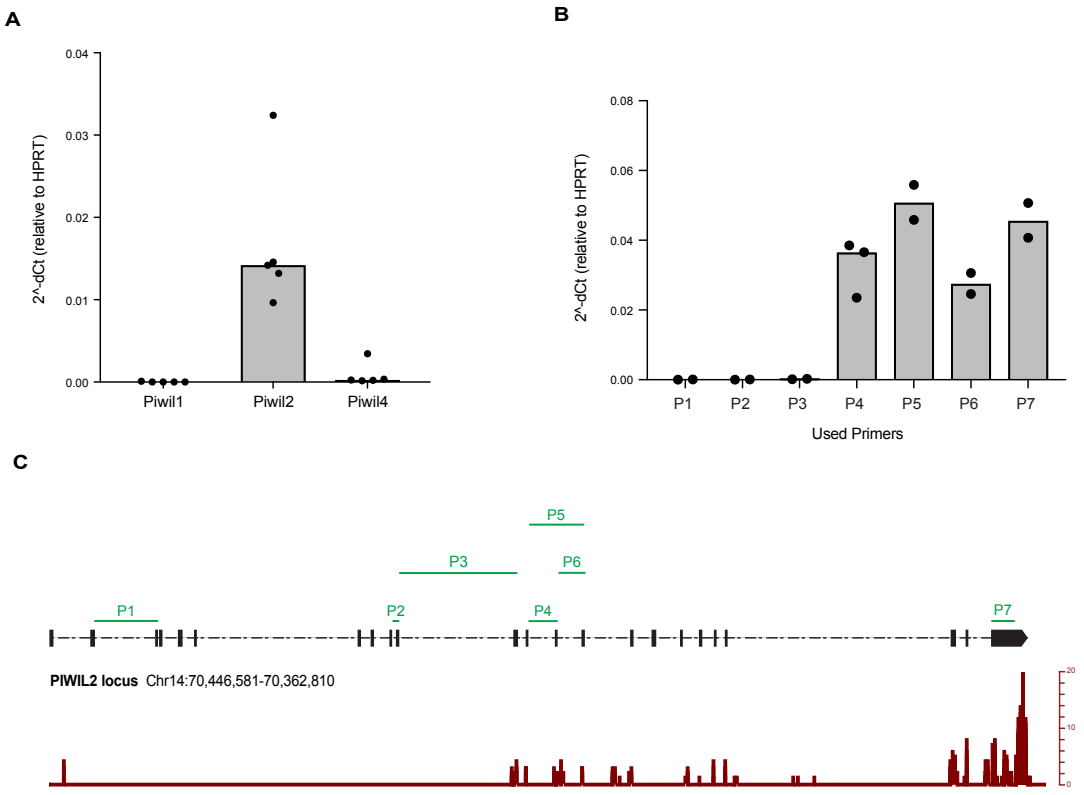

Figure 7: Expression of PIWI family members in alveolar macrophages. (A) Expression of the three murine PIWI family members in alveolar macrophages analyzed by RT-qPCR. (B) RT-qPCR amplification products of primers mapping along the Piwil2 locus (shown in green in C) in alveolar macrophages. For A and B, values are presented as the median of biological replicates and are normalized to HPRT. (C) RNA-Sequencing reads mapping to the Piwil2 locus (depicted in black. Tick marks represent exons) in alveolar macrophages.

6.2.2 *GM-CSF Regulates Piwito Expression in vivo and in vitro*

The cytokine GM-CSF has been shown to induce the proliferation of alveolar macrophages in vitro (Akagawa et al., 1988; Chen et al., 1988). To approach the question whether Piwito plays a role in the self-renewal of alveolar macrophages, we tested if its expression can be influenced by GM-CSF signaling. We stimulated AM with recombinant GM-CSF in culture and tested for Piwito expression at different time points after stimulation. As expected, we observed an increase in Piwito mRNA upon GM-CSF stimulation (Figure 8A). Consistent with this, we also saw an impact on Piwito expression when blocking GM-CSF signaling in vivo. To impair GM-CSF signaling in vivo, we delivered a neutralizing anti-GM-CSF antibody or an isotype control directly into the trachea of mice (intratracheal injection) every other day for 5 days. Two days after the last injection, we sorted alveolar macrophages and evaluated the expression of Piwito by RT-qPCR. In concordance with our previous observation, we saw a decrease in Piwito expression in the samples where GM-CSF signaling was impaired (Figure 8B).

These observations provide strong evidence for a regulation of Piwito levels in AM by the growth factor GM-CSF, in vitro and in vivo. Additionally, they support the hypothesis for a role of Piwito in alveolar macrophage self-renewal.

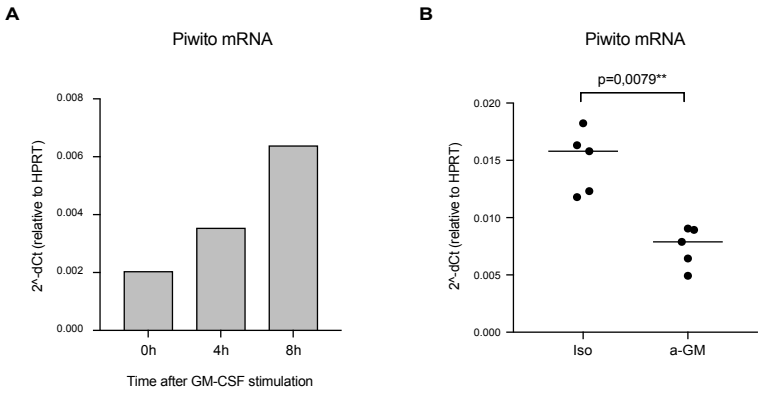

Figure 8: Regulation of Piwito expression by GM-CSF. (A) Piwito mRNA levels in cultured alveolar macrophages at different time points after GM-CSF stimulation (20ng/mL). Values are normalized to HPRT (B) Piwito mRNA in sorted alveolar macrophages after intratracheal treatment with a GM-CSF-neutralizing antibody or an isotype control. Values are normalized to HPRT. The line shows the median.

6.2.3 *Deficiency of Piwito Affects Alveolar Macrophage Self-Renewal in Steady State*

To elucidate the role of Piwito in alveolar macrophages we used a mouse model with a genetic mutation that impedes the expression of the Piwil2 pro-

tein (Di Giacomo et al., 2013). The mutation is the excision of exon 21 of the Piwil2 gene (Figure 9A). This results in an out-of-frame splicing that leads to a premature stop codon. Prematurely stopped mRNA transcripts are eliminated by nonsense-mediated decay (NMD), resulting in a failure to express the concerned gene. Importantly, since this mutation affects the C-terminal end of Piwil2, Piwito transcripts should be equally affected. This mouse model hereafter referred to as Piwito-KO, represents a model to study mechanisms in the absence of the full-length Piwil2 as well as the N-terminal truncated isoform Piwito. As expected, Piwito transcripts were absent from freshly harvested alveolar macrophages in Piwito-KO mice (Figure 9B).

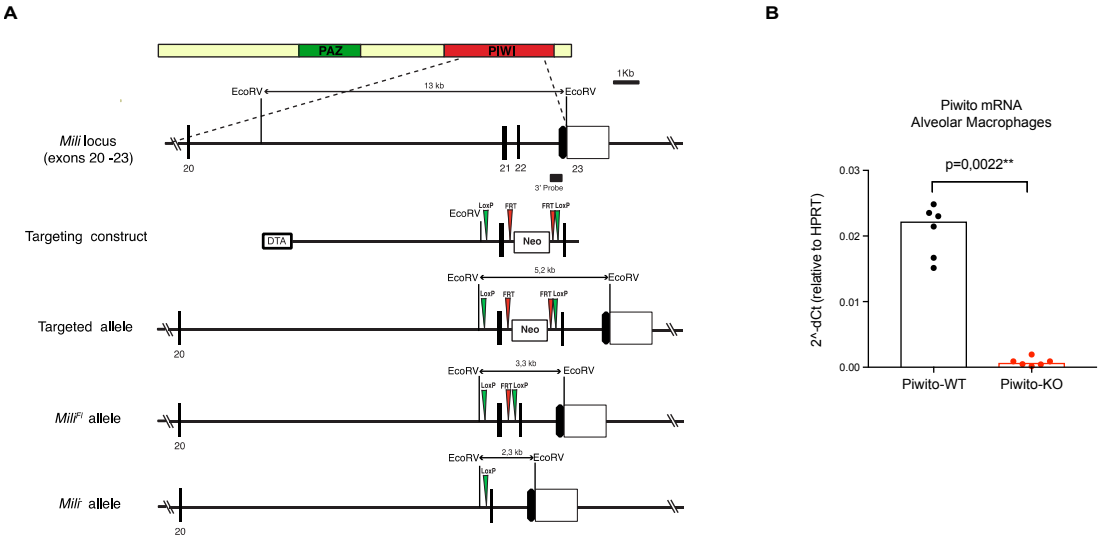

Figure 9: A mouse model deficient for Piwito. (A) Representation of the murine Piwil2 locus and the targeting strategy used to generate the mice used in this study. Adapted from (Di Giacomo et al., 2013). (B) Piwito mRNA levels in freshly harvested alveolar macrophages of Piwito-WT or –KO mice. Values are pooled from two independent experiments and the line represents the median.

To gain more information on alveolar macrophage self-renewal in the absence of Piwito, we measured the percentage of cycling cells within the alveolar compartment of Piwito-KO or –WT mice in vivo by flow cytometry. More precisely, we quantified the percentage of AM that incorporated intra-peritoneally injected EdU it into newly synthetized DNA in a time frame of four hours. Interestingly, we found that EdU+ alveolar macrophages were significantly reduced in the absence of Piwito (Figure 10A). This observation was further validated in vitro by a colony formation assay (CFA) made with freshly harvested Piwito-KO AM and WT controls. In concordance with our previous result, alveolar macrophages from Piwito-KO mice formed fewer colonies than WT controls (Figure 10B). Surprisingly, however, we found that this self-renewal defect did not affect alveolar macrophages in vivo (Figure 10C).

These results suggest that alveolar macrophages deficient for Piwito have a diminished self-renewal capacity when compared to WT alveolar macro-

phages. However, the extent of this defect is not sufficient to affect AM homeostasis in the steady state, as AM numbers remain unchanged in the absence of Piwito.

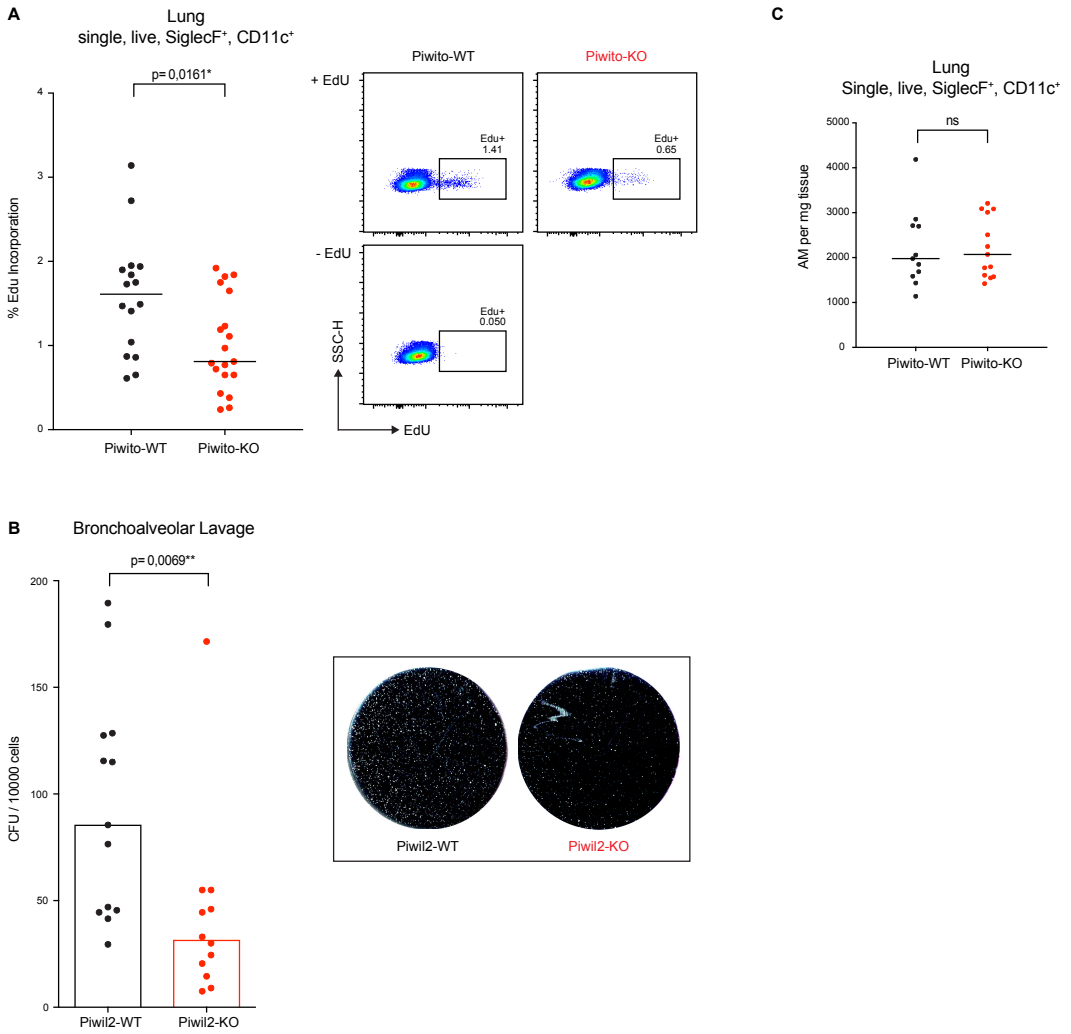

Figure 10: Proliferation Assays on Piwito-WT and –KO alveolar macrophages. (A) Percentage of EdU+ cells in the alveolar macrophage compartment (SiglecF+, CD11c+) of Piwito-WT or –KO mice. EdU was injected intraperitoneally 4 hours before harvesting the lungs. On the right, representative FACS plots of EdU+ and EdU- alveolar macrophages in Piwito-WT and –KO mice. (B) Colony Formation Assay of alveolar macrophages from individual mice. Values represent the colony counts for 1000 initially plated cells. On the right: representative pictures of a CFA dish of Piwito-WT and Piwito-KO alveolar macrophages. (C) Absolute numbers of alveolar macrophages (single, live, SiglecF+, CD11c+) normalized to tissue weight in the lungs of Piwito-WT and –KO mice. Values in each graph are a pool of three independent experiments. The line represents the median. P-values were calculated using the Mann-Whitney Test.

6.2.4 *Piwito Deficiency Affects Macrophage Self-Renewal Upon Challenge*

Although alveolar macrophages showed compromised proliferation in vivo and in vitro, they were still capable of maintaining homeostatic population numbers. It is known that upon challenge, macrophage proliferation can increase to compensate for the lost (or cope with the higher demand) of macrophages (Hashimoto et al., 2013; Jenkins et al., 2013; Sieweke and Allen, 2013). Therefore, we decided to check whether Piwito deficiency impacts the proliferative response of alveolar macrophages in response to a challenge. For this purpose, we established an assay in which an experimentally-induced insult induces proliferation beyond homeostatic levels in alveolar macrophages. Specifically, we depleted alveolar macrophages using liposome-encapsulated clodronate. Clodronate is a drug that causes apoptosis upon accumulation in the cytoplasm. It cannot pass cell membranes and therefore, as it is delivered encapsulated in liposomes, it affects only phagocytic cells. Because we specifically wanted to deplete alveolar macrophages, we delivered the clodronate liposomes intratracheally.

We followed alveolar macrophage numbers after clodronate depletion by flow cytometry and saw an efficient and quick depletion. Two days after treatment, the alveolar macrophage population was reduced to approximately 15% of the initial numbers. The experiment also revealed that alveolar macrophages recover with very slow kinetics, coming back to normal numbers only fourteen days post-depletion (Figure 11A). In parallel, we measured the proliferation rates of AM by means of EdU incorporation and Ki67 intracellular staining. Consistently between both proliferation readouts, basal proliferation of alveolar macrophages increased gradually after depletion to reach a peak at four to six days post-depletion. After this peak, proliferation rates decrease again until basal values were re-established at around ten days post-depletion (Figure 11B). This experiment showed that alveolar macrophages respond to clodronate-driven depletion of the niche by a compensatory proliferation and that physiological cell numbers are restored two weeks post-depletion.

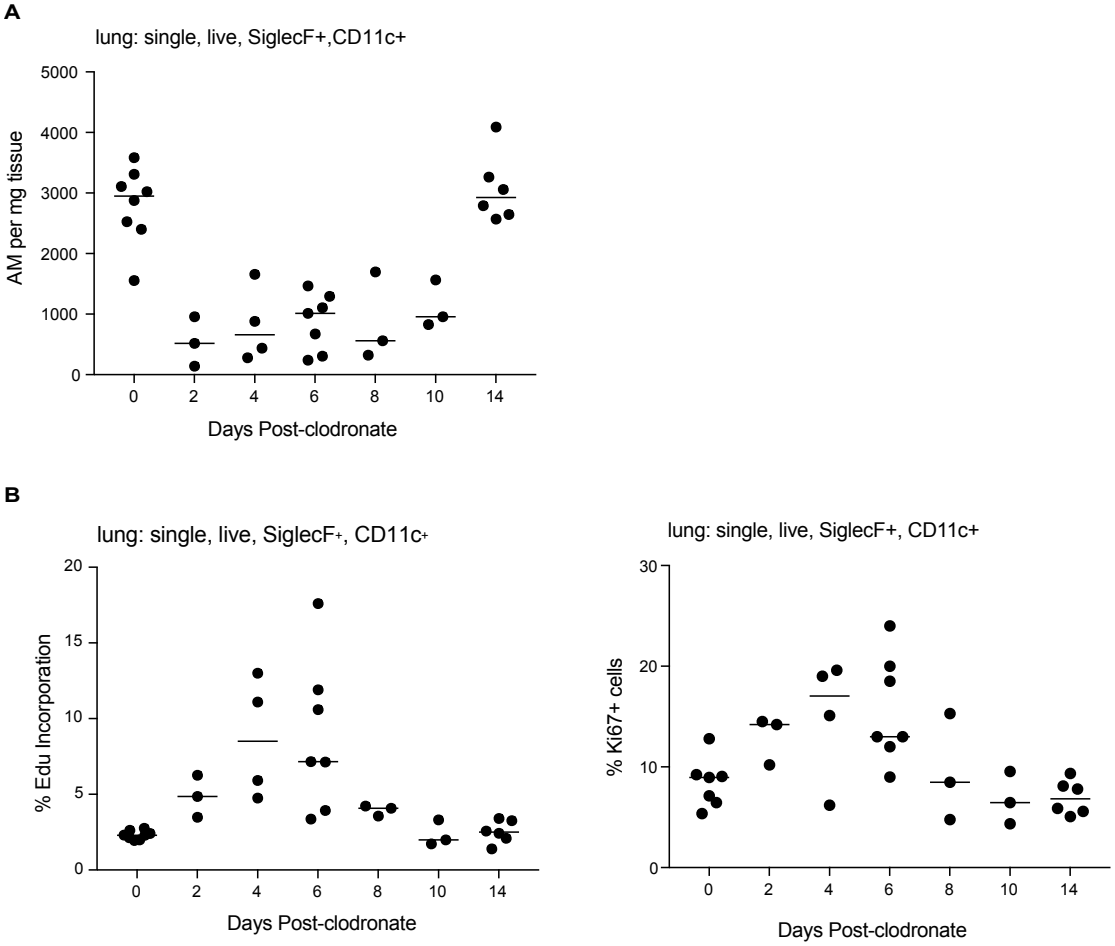

Figure 11: Kinetics and proliferation of alveolar macrophages following intratracheal clodronate treatment. Liposome-encapsulated clodronate was given directly into the trachea of WT mice and analysis of the alveolar compartment (SiglecF+, CD11c+) was done by flow cytometry at different time points post-treatment. (A) Absolute numbers of alveolar macrophages in WT mice upon clodronate treatment. Values were normalized to tissue weight. (B) Percentage of EdU+ and (C) Ki67+ alveolar macrophages in WT mice upon clodronate treatment. Values are shown as a pool of three independent experiments. The line represents the median.

Having established a system in which alveolar macrophages react to a challenge by proliferation, we wondered if this proliferation would be affected by the absence of Piwito. We used intratracheal administration of clodronate to deplete alveolar macrophages of mice deficient for Piwito or counterpart controls and checked the percentage of proliferating cells six days post-depletion. We chose this time point because it coincided with the proliferation peak in our previous experiment (Figure 11B). Consistent with our observations so far, Piwito-deficient alveolar macrophages showed a reduced compensatory proliferation in comparison to WT controls, reflected by a decrease in the percentage of Edu+ cells six days post-depletion (Figure 12A). However, despite the difference in the percentage of cycling cells, Piwito-KO alveolar macrophages were able to repopulate the depleted niche with efficiency and kinetics comparable to WT controls (Figure 12B).

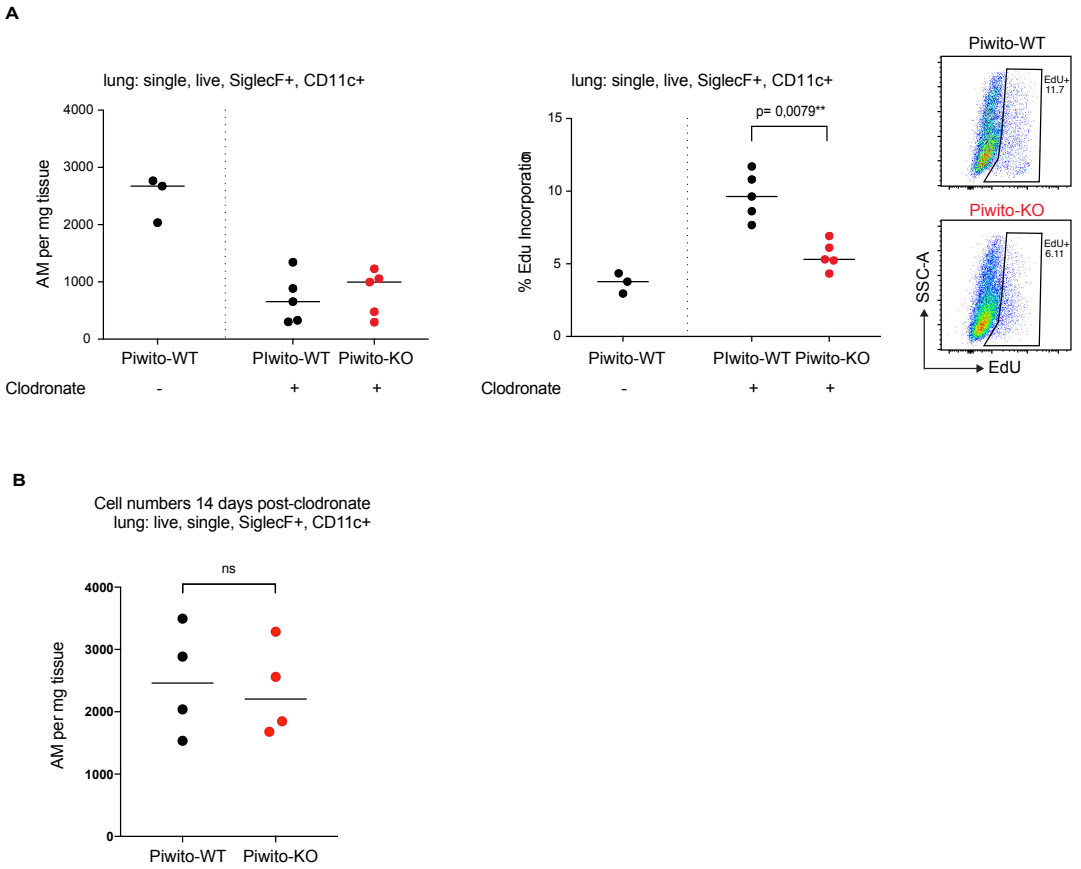

Figure 12: Intratracheal clodronate treatment on Piwito-WT and –KO mice. Liposome-encapsulated clodronate was given directly into the trachea of Piwito-WT and –KO mice and analysis of the alveolar compartment (SiglecF+, CD11c+) was done by flow cytometry six days post-treatment. (A) Absolute numbers of alveolar macrophages six days after clodronate treatment. Values were normalized to tissue weight. (B) Percentage of EdU+ alveolar macrophages six after clodronate treatment. On the right, representative FACS plots of EdU+ and EdU- alveolar macrophages in Piwito-WT and –KO mice. Values are shown as a pool of two independent experiments. P-values were calculated using the Mann-Whitney Test (C) Absolute numbers of alveolar macrophages in Piwito-WT and –KO mice fourteen days after clodronate treatment. In all graphs, the line represents the median.

This last result is analogous to our observations on Piwito-deficient AM in the steady state. Upon clodronate depletion, we were able to detect a proliferation defect, but it did not disturb the re-establishment of homeostasis by alveolar macrophages.

6.2.5 *Piwito-Deficient AM are Able to Repopulate an Empty Lung Niche by Local Self-Renewal*

It has been reported that clodronate-driven depletion of alveolar macrophages induces recruitment of circulating monocytes (Everhart et al., 2004). Recruited monocytes could contribute to the alveolar pool and thus to the reestablishment of the depleted macrophage population. In that case, local self-renewal would be a redundant mechanism for the recovery of the AM population, and a proliferation defect may not affect the repopulation of the alveolar niche. Based on this reasoning, we hypothesized that, in our previous experiment, monocyte recruitment masked the inability of Piwito-KO AM to reestablish homeostatic population numbers after depletion. To test this hypothesis, we designed an experiment in which monocyte recruitment cannot contribute to the repopulation of the alveolar niche after depletion.

Our approach was an adoptive transfer of alveolar macrophages into the lungs of mice that are deficient for the GM-CSF receptor (Csf2rβ-KO mice, (Robb et al., 1995). Because GM-CSF signaling is indispensable for alveolar macrophage establishment and development (Guilliams et al., 2013; Schneider et al., 2014), lungs of Csf2rβ-KO mice are essentially devoid of alveolar macrophages (Happle et al., 2014; Schneider et al., 2014). Therefore, they represent an easily accessible, empty niche that can be repopulated by cells bearing functional GM-CSF receptors (Happle et al., 2014; Suzuki et al., 2014; van de Laar et al., 2016). Evidently, cells that seed the lungs of Csf2rβ-KO mice have to fill up the niche by their own proliferation, since host monocytes are present but incapable of colonizing the lung.

We seeded the lungs of CD45.1 Csf2rβ-KO newborn mice with CD45.2 Piwito-WT or –KO alveolar macrophages by intranasal delivery of the cells. Four weeks after transplantation, we examined the lungs of the mice and found no significant difference in the repopulation efficiency of Piwito-WT or –KO alveolar macrophages (Figure 13). This result suggests that Piwito deficiency does not impair the repopulation capabilities of alveolar macrophages, even if they have to act independently of monocyte recruitment.

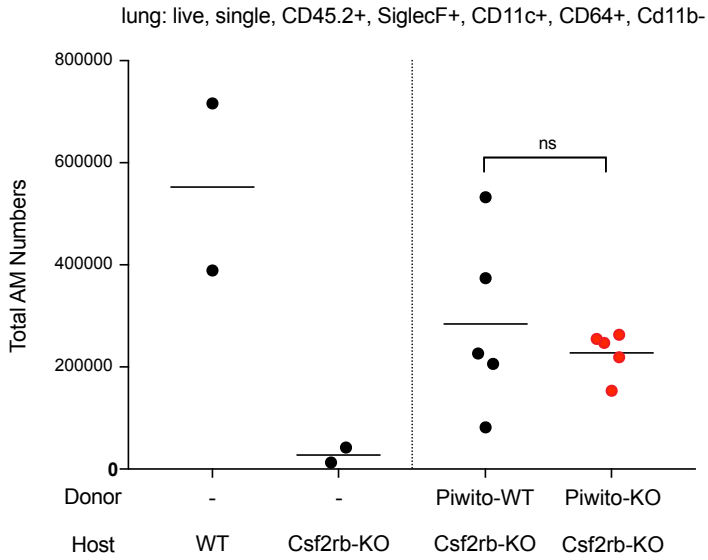

Figure 13: Adoptive transfer of Piwito-WT and –KO alveolar macrophages into Csf2rβ-KO mice. Total number of alveolar macrophages recovered by BAL 4 weeks post-transplantation. Values represent total numbers calculated by flow cytometry. One representative experiment is shown. The line represents the median. P-values were calculated using the Mann-Whitney Test.

6.2.6 *Piwito Regulates the Lifespan of Alveolar Macrophages in culture*

The two most prominent examples of PIWI function are germline maintenance and whole body regeneration. Remarkably, both functions are exerted in the only metazoan cells that can be considered to have an ageless genome that is not subjected to senescence. Therefore, based on the hypothesis that PIWI function on the maintenance of genome integrity is linked to the regulation of cellular lifespan, we theorized that Piwito affects alveolar macrophage homeostasis only on the long-term, maybe even beyond physiological lifespan.

We thus asked whether Piwito deficiency restricts the lifespan of alveolar macrophages. To answer this question, we harvested alveolar macrophages from mice deficient for Piwito and their WT counterparts and established AM cultures of each mouse individually. We followed and documented the growth of the cultures for 140 days. At around 65 days of culture, we noticed a difference in population doubling numbers between both genotypes, with a proliferation disadvantage for Piwito-KO alveolar macrophages. This difference increased with time, as WT cells steadily increased in numbers whereas Piwito-deficient cells progressively reduced their division rate (Figure 14A,B). Remarkably, by 140 days in cultures, all Piwito-KO cell lines had stopped dividing (Figure 14C), as judged by their "static" permanence in the cell culture dishes without further growth. This notable experiment showed that Piwito-KO alveolar macrophages fail to maintain their populations after a prolonged

expansion in culture and thus proposes a role for Piwito in lifespan regulation in alveolar macrophages.

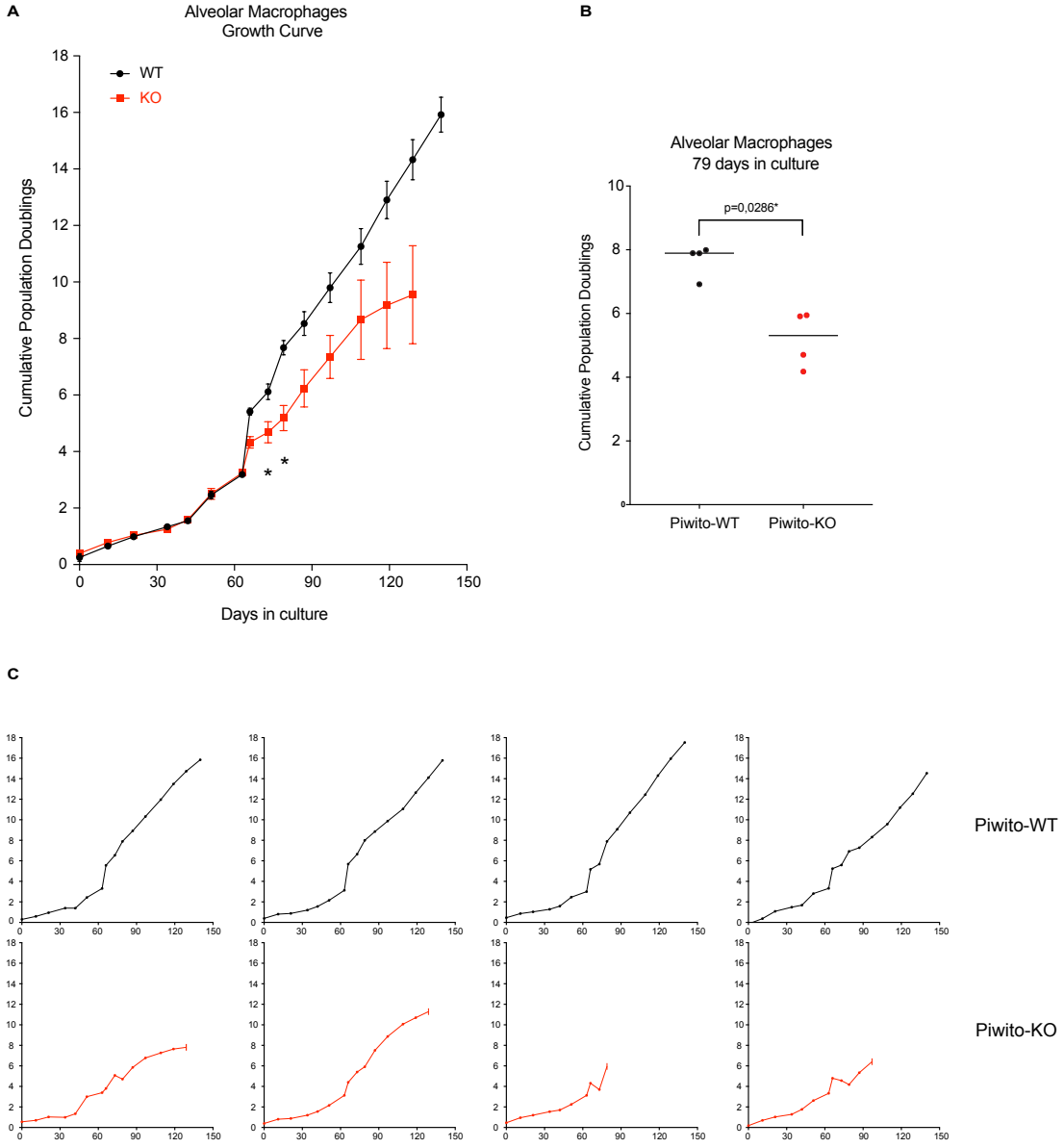

Figure 14: Long-term culture of Piwito-WT or –KO alveolar macrophages. (A) Cell growth represented as cumulative populations doublings. Data points represent the mean of biological replicates. Error bars represent the SEM. (B) Values for population doublings in individual mice after 79 days in culture. The line represents the median. (C) Growth curve plotted for each individual mouse for clarity. For all Piwito-KO mice, the tick mark at the end indicates the time when the culture stopped growing. One representative experiment is shown. P-values were calculated using the Mann-Whitney Test.

In an attempt to understand the mechanism underlying lifespan regulation by Piwito, we extracted RNA from the cells at day 87 of culture and checked by RT-qPCR for the expression of LINE1 (L1) transposable element component ORF2. ORF2 stands for 'open reading frame 2' and is one of the two regions

that encodes for a protein in active LINE1 (L1) element transcripts (Dombroski et al., 1991). ORF2 encodes for a protein with DNA endonuclease and reverse transcriptase activity that is required for L1 retrotransposition (Feng et al., 1996; Mathias et al., 1991; Moran et al., 1996). Importantly, ORF2 (together with ORF1) can also promote the retrotransposition of non-autonomous TEs such as short interspersed elements (Hancks et al., 2011; Raiz et al., 2011), or even of mRNAs (Esnault et al., 2000; Wei et al., 2001).

We observed an increase of the ORF2 transcript in our Piwito-KO cultures when compared to WT cells (Figure 15A). Accordingly, another primer pair targeting the L1 transcript outside of the open reading frames also revealed increased expression in Piwito-KO cells compared to WT (Figure 15B). Although these results are preliminary, they suggest that Piwito may prolong the lifespan of alveolar macrophages by maintaining low levels of retro-transposition in the cells.

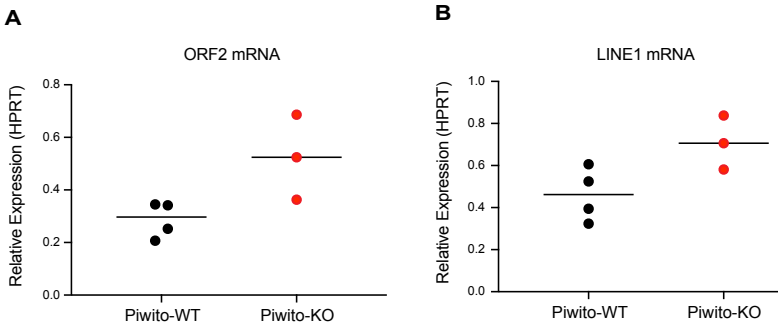

Figure 15: Expression of LINE-1 element transcripts in cultured Piwito-KO or-WT alveolar macrophages. LINE1 element transcripts analyzed by RT-qPCR in alveolar macrophages at day 87 of culture. Values are normalized to HPRT. One representative experiment is shown. The line represents the median.

6.3 MAFB IN THE REGULATION OF PIWI EXPRESSION

6.3.1 *MafB Directly Regulates Piwito in MafDKO Macrophages*

In the last two sections, we have collected evidence for a role of Piwito in macrophage self-renewal. Our laboratory has shown that the myeloid transcription factor MafB is a negative regulator of macrophage self-renewal because it binds and represses a group of genes that collectively enable macrophage self-renewal (Soucie et al., 2016). Given the reported regulation of PIWI proteins by the Maf homolog Traffic Jam in Drosophila (Saito et al., 2009), we hypothesized that MafB negatively regulates Piwito expression as part of its broader role in the inhibition of macrophage self-renewal.

To test this hypothesis, we took advantage of a system established in our laboratory that allows the inducible expression of MafB in MafDKO macrophages. In this system, a Flag-tagged MafB operates under the control of a tetracycline (TET)-inducible promoter in cells that carry a reverse tetracycline-controlled transactivator (rtTA). Hence, the system allows the inducible and reversible expression of MafB in the presence of doxycycline (Figure 16A). Additionally, the Flag-MafB vector (or its empty vector control) expresses GFP constitutively to track their expression in the host cells (Figure 16B).

Using this system, we saw that upon overexpression of MafB, Piwito expression decreased in MafDKO macrophages when compared to the EV control (Figure 16C). This anti-correlation suggested a regulation of Piwito by MafB in a similar fashion to other genes controlling macrophage self-renewal. To assess whether MafB is directly bound to Piwito, we interrogated our laboratory ChIP-Sequencing data on genome-wide binding of MafB in WT macrophages. As expected, we found a peak in the Piwil2 locus, in the region corresponding to the intron before the first exon coded in the Piwito transcript. This peak coincided with H3K4m3 marks in MafDKO macrophages that were absent from WT controls. Therefore, we concluded that MafB directly binds and represses Piwito in quiescent macrophages (Figure 16D).

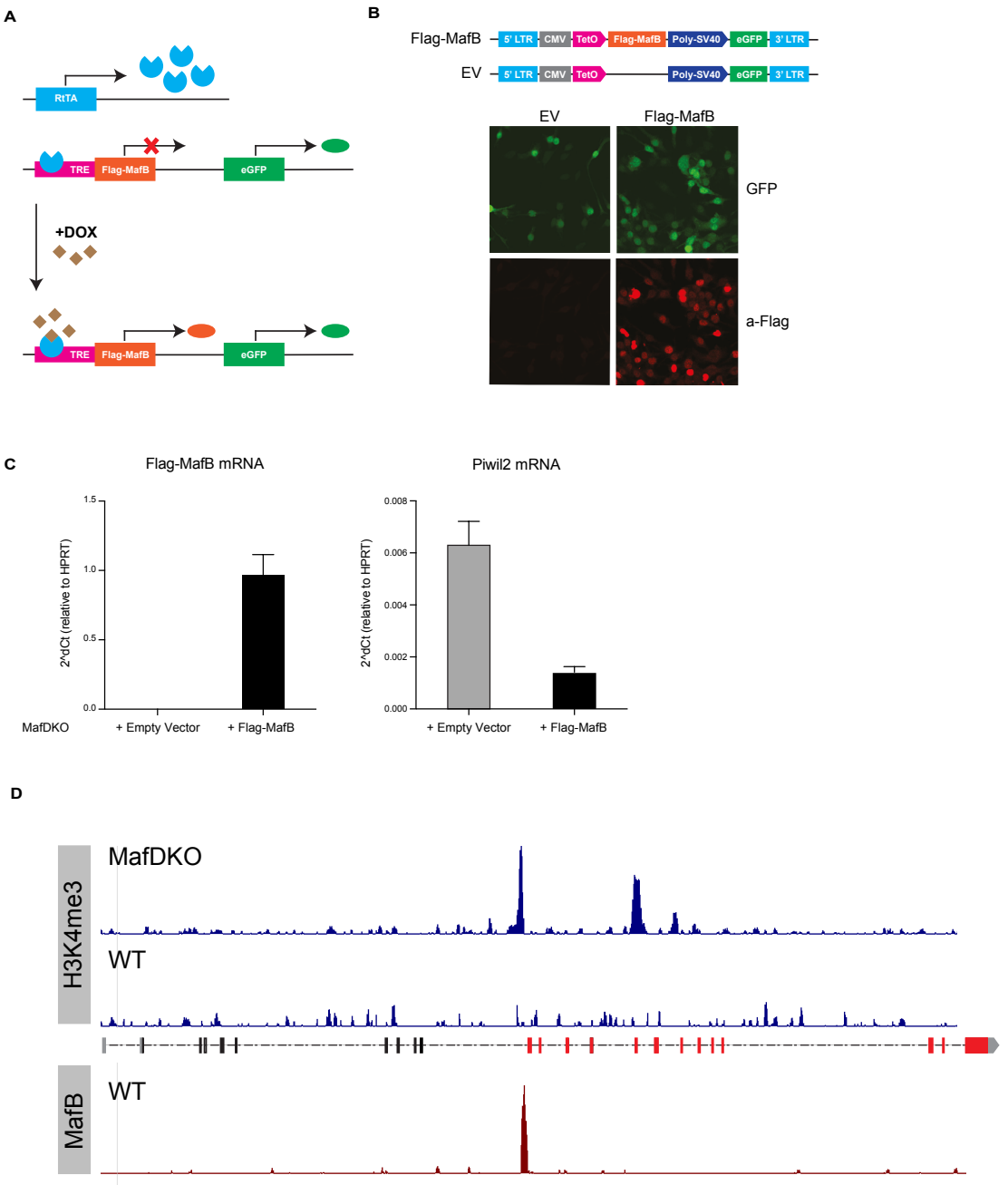

Figure 16: Regulation of Piwito expression by MafB. (A) Scheme of the system used for Doxycycline-induced MafB expression. (B) Scheme of the Flag-MafB and control constructs and validation of their expression (GFP) in host cells and of the induction of Flag-MafB. (C) RT-qPCR on MafDKO macrophages over-expressing Flag-MafB or an empty vector control. All cells were treated with 1ug/mL of Doxycycline for 8 hours prior to RNA extraction. Values are normalized to HPRT. Data are presented as the mean of three independent experiments. Error bars indicate SD. (D) Genomic region corresponding to the Piwil2 locus in MafDKO or WT macrophages with ChIP-Sequencing tracks as labeled. In the scheme for the Piwil2 locus, tick marks represent exons. Exons that are present in the Piwito transcript are highlighted in red.

6.3.2 *MafB expression anti-correlates with Piwito expression in vivo*

We wondered if MafB also regulates Piwito expression in vivo. To check this, we compared Piwito expression of AM with two macrophage populations that, as opposed to AM, are known to express high levels of MafB: peritoneal macrophages (PM) and bone marrow-derived macrophages (BMDM). Interestingly, we found that PM and BMDM, in contrast to AM, express low levels of Piwito mRNA (Figure 17A). Additionally, we found that the disruption of GM-CSF signaling in vivo by the intratracheal administration of a GM-CSF-neutralizing antibody, a condition that down-regulates Piwito expression levels (see Section 2.2.), increases the levels of MafB in alveolar macrophages (Figure 17B). These observations, in conjugation with the data in MafDKO macrophages, strongly suggest that MafB regulates Piwito levels in vivo as well.

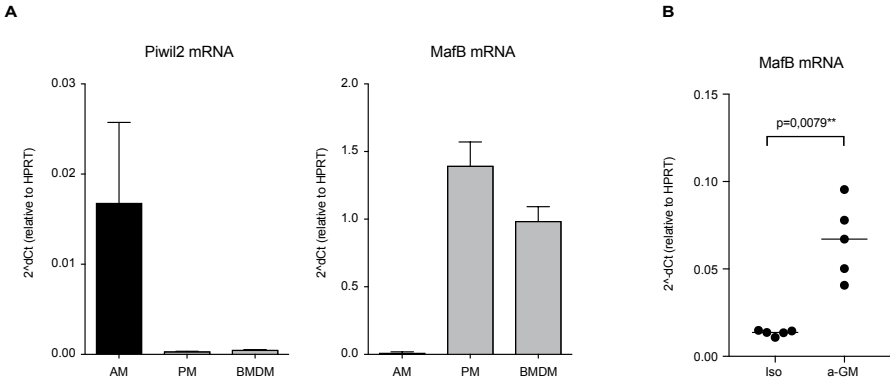

Figure 17: Expression of Piwito and MafB levels in different macrophage populations (A) Piwito and MafB mRNA transcripts in fresh alveolar macrophages (AM), sorted peritoneal macrophages (PM) and bone marrow-derived macrophages (BMDM) by RT-qPCR. (B) MafB mRNA in sorted alveolar macrophages after intratracheal treatment with a GM-CSF-neutralizing antibody or an isotype control. Values are normalized to HPRT and represented as the median of biological replicates. One representative experiment is shown. P-values were calculated using the Mann-Whitney Test.

6.4 MAFB EXPRESSION INCREASES IN MICROGLIA OVER TIME

We have shown that Piwito plays a role in alveolar macrophage proliferation and that it is a direct target of MafB. Interestingly, another study in which I participated during my Ph.D. (Matcovitch-Natan et al., 2016), discovered that MafB levels increase in macrophages from early development to the adult mouse. This study was focused on the resident macrophages of the brain, microglia, and desired to reveal the dynamic changes occurring in gene expression and chromatin structure during development and maturation of microglia. For this purpose, microglia were collected at different time points during de-

velopment or adulthood and RNA-Sequencing, ChIP-Sequencing on histone modifications and ATAC-Sequencing was performed on the obtained samples.

Collectively, those studies showed that microglia drive different transcriptional programs as they develop into adult cells. Those programs run in concordance with the surrounding brain development and allow them to support brain development and function in an age-specific manner. Pre-natal microglia display a transcriptome enriched in pro-proliferative genes, suggesting that their main activity is to populate the freshly colonized niche. Microglia around birth drive processes that involve neurogenesis or cytokine secretion, in concordance with their supportive role on neural migration and maturation. Finally, adult microglia are characterized by a mature canonical macrophage profile, necessary to fulfill functions of immune surveillance and tissue maintenance.

Interestingly, MafB was one of the top-upregulated transcription factors in the transition from young (around birth) microglia to adult microglia. Comparison of adult MafB-KO or –WT microglia suggested that MafB participates in the immune maturation of microglia. Additionally, MafB increase from developing to adult microglia may also explain the dynamic shift from a pro-proliferative state around the embryonic period to a more quiescent one in the adulthood. Thus, these results suggest that MafB control of macrophage proliferation may also apply to microglia.

6.5 SIRTUINS IN THE CONTROL OF MACROPHAGE SELF-RENEWAL

Another study in which I participated during my Ph.D. (Imperatore et al., 2017) revealed a role for the histone deacetylase SIRT1 in macrophage self-renewal. This study showed that shRNA-mediated knockdown of SIRT1, genetic deletion by CRISPR/Cas9 or the use of the natural sirtuin inhibitor nicotinamide (NAM) restricted the proliferation of MafKO and alveolar macrophages in vitro. These observations were validated in vivo since we observed that steady state and cytokine-induced proliferation of alveolar and peritoneal macrophages were negatively affected by the administration of NAM.

Mechanistically, SIRT1 was shown to act by a double strategy of impeding cell cycle progression and inducing cell cycle arrest. Gene expression analysis revealed that the administration of NAM blocks cell cycle progression beyond G1 and down-regulates most of the genes that have been shown to drive macrophage proliferation, such as Myc and Klf2 (Soucie et al., 2016). Consistent with this, SIRT1 downregulation was shown to inactivate E2F1, an important transcription factor in cell cycle control. Finally, knockdown of SIRT1 or its inhibition by NAM was shown to induce cell cycle arrest by the activation of the transcription factor FoxO1. This study suggests that macrophages control proliferation by various, redundant mechanisms. The highly conserved role

of sirtuins in longevity may also involve replicative lifespan regulation, stress response, and control of DNA stability.

Part III

DISCUSSION

7

DISCUSSION

7.1 CONTEXT

PIWI proteins are expressed predominantly in self-renewing cells and their best-described function is the maintenance of genome integrity through the repression of transposable elements. PIWI expression is functionally required in several stem cell compartments in the invertebrate world. However, in mammalians, PIWI function has only been observed in the male germline. Although expression of PIWI proteins or piRNAs has been reported in mammalian somatic cells, reliable functional studies are missing. To the contrary, in two cases, exhaustive functional studies have concluded that PIWI function is dispensable for the studied stem cell systems: the mouse hematopoietic system (Nolde et al., 2013) and the development of murine induced pluripotent stem cells (Cheng et al., 2014). Therefore, and despite repeated suggestive observations, evidence for a somatic function of PIWI proteins in mammals had remained absent prior to my study.

The specificity of PIWI expression reflects a stringent regulation. To date, little is known about transcriptional regulators of PIWI proteins and no conserved regulatory mechanism has been reported. Piwi gets transiently upregulated in the Drosophila gut during episodes of regeneration in response to the JAK/STAT signaling pathway (Sousa-Victor et al., 2017). In the ovarian soma of Drosophila, the Maf homolog Traffic Jam regulates the expression of Piwi and the 3'UTR of its mRNA transcript serves as a source of piRNAs (Saito et al., 2009). In mammals, the only known regulator of PIWI function is the transcription factor A-Myb, which induces the transcription of piRNAs, Piwil1 and other effector proteins of the PIWI/piRNA pathway in the mouse testis (Li, Roy, Dong, Bolcun-Filas, Wang, Han, Xu, Moore, Schimenti, Weng and Zamore, 2013).

Taken together, the current literature supports a conserved role for PIWI in self-renewal in somatic and germline tissues of invertebrate animals. In mammals, however, functional evidence of PIWI function is available exclusively for the germline. Furthermore, information on the transcriptional regulation

of the PIWI/piRNA pathway is very limited, with no conserved regulatory mechanism reported to date.

7.2 RECAPITULATION OF MAIN RESULTS

Given the highly conserved role of the PIWI/piRNA pathway in the process of self-renewal and its regulation by the Maf homolog Traffic Jam in Drosophila, we decided to analyze whether PIWI proteins play a role in macrophage self-renewal.

We were able to show that MafB an c-Maf deficient (Maf-DKO) bone marrow-derived macrophages, which possess the capacity to self-renew, express the transcript of a N-terminal truncated form of the mouse PIWI protein Piwil2. This shorter isoform, which we have named 'Piwito', comprises exon 11 to 23 of the full-length transcript. Piwito expression is positively regulated by the growth factor M-CSF in Maf-DKO macrophages and its down-regulation compromises the proliferation capacity of Maf-DKO macrophages. Upon knock-down of Piwito, Maf-DKO macrophages showed a slower growth in liquid culture, a reduction of colony forming capacity and a decrease in the percentage of cells that enter the S-Phase of the cell cycle.

Furthermore, we were able to extend our observations to genetically unmodified, self-renewing alveolar macrophages (AM). We could show that alveolar macrophages express the Piwito transcript as well, which encompasses exons 11 to 23 of the full-length Piwil2 transcript. Similar to our observations on Maf-DKO macrophages, Piwito expression in alveolar macrophages is regulated in vivo and in vitro by a growth factor, in this case, GM-CSF, which is required for AM maintenance.

Using mice with a genetic mutation that impedes the expression of Piwito and full-length Piwil2, we discovered that alveolar macrophage proliferation in vivo is compromised in the absence of Piwito. Piwito-KO alveolar macrophages displayed a lower percentage of cycling cells in the steady state or during recovery from experimental depletion compared to WT controls. Piwito-KO alveolar macrophages also formed less colonies in a colony formation assay. This proliferation defect, however, did affect the absolute numbers of alveolar macrophages in vivo, indicating that the proliferation defect can be compensated.

In addition, the repopulation of an empty alveolar niche also occurred in the absence of Piwito. Piwito-KO alveolar macrophages were still able to re-establish normal numbers upon clodronate-dependent macrophage depletion. Similarly, upon intranasal adoptive transfer, they were able to seed and fill up the niche of mice deficient for the GM-CSF receptor, which lack alveo-

lar macrophages. This last experiment proved that Piwito-deficient alveolar macrophages are able to fill up an empty niche by local self-renewal, since monocytes of GM-CSF receptor-deficient mice are not capable to differentiate into alveolar macrophages.

Strikingly, however, we observed that in contrast to unmodified alveolar macrophages, Piwito-KO alveolar macrophages could not be maintained in long-term culture. 65 days old Piwito-KO AM cultures started to show a decrease in their growth rate and fewer population doublings. This difference between Piwito-KO and WT cultures became more pronounced over time until eventually all tested Piwito-KO alveolar macrophage lines stopped dividing, while WT cells continued their growth without obvious abnormalities.

Finally, we could show that the transcription factor MafB directly binds and represses an intragenic promoter in the intron 10 of the Piwil2 locus in WT bone marrow-derived macrophages. Due to its position, we hypothesized that it is the promoter of Piwito. Consistent with this notion, re-expression of MafB in MafDKO macrophages down-regulated Piwito expression. In addition, we could show that there is a negative correlation between the expression of Piwito and MafB transcripts in vivo. Macrophage populations with low MafB levels express high levels of Piwito, whereas populations with high MafB levels express low levels of Piwito. Importantly, Piwito expression in alveolar macrophages was decreased upon experimental disruption of GM-CSF signaling, a condition that up-regulates MafB levels in the cells.

7.3 INTERPRETATION OF RESULTS

7.3.1 *Macrophages express an N-truncated isoform of Piwil2*

Our data indicate that Piwito is not derived from splicing of the full length Piwil2 transcript, but rather transcribed directly from an intragenic promoter localized in the intron 10 of the Piwil2 locus. The expression of Piwil2 as a set of N-truncated isoforms of different lengths has been reported for human and mouse cancer cells. Among the reported isoforms, the one that could correspond to Piwito (Exon 11-23) is the most frequent one, common to the majority of cancer types analyzed (Chen et al., 2007; Ye et al., 2010; Gainetdinov et al., 2014; Liu et al., 2017). Moreover, a promoter in the intron 10 of the human Piwil2 locus has been cloned and described (Liu et al., 2017). Of note, these studies have shown that a Piwil2 transcript of the length of Piwito is translated into a detectable protein. Consitent with our results on macrophages, the short isoform that could correspond to Piwito plays a role in the proliferation of mouse and human cancer cells, since its downregulation suppresses

the growth of cancer cells and its overexpression promotes the proliferation of mouse bone marrow progenitors (Chen et al., 2007; Ye et al., 2010).

Given the fact that Piwito starts at exon 11 of the full length Piwil2 transcript, it lacks the N-terminal domain and probably also a functional PAZ domain, because the PAZ domain in the full-length Piwil2 starts with the last amino acids of exon 10 (NCBI Gene Database). Assuming that the two remaining protein domains encoded in the Piwito transcript are functional, Piwito would consist of a protein comprising a PIWI and a MID domain. Both domains would ensure a correct recognition and binding of the 5'end of a piRNA as well as correct base pairing of the bound RNA with its target. Additionally, the ribonuclease activity of PIWI proteins is encoded in the PIWI domain, suggesting that Piwito may retain slicer activity towards its targets. The PAZ domain provides a binding pocket for the 2'-O-methylated 3'end of piRNAs. Mutations on the amino acids that define this binding pocket reduce piRNA load but do not abolish it (Matsumoto et al., 2016). Similarly, it has been shown that the Argonaute protein Ago2, which shares all protein domains with PIWI proteins, is able to associate to with small RNAs and to cleave them in the absence of a PAZ domain (Gu et al., 2012). It is thus valid to speculate that Piwito is able to bind small RNAs and cleave small RNA-bound targets.

The structure of Argonaute proteins is organized in two lobes connected by linker sequences, one comprising the N-PAZ domains and the other one comprising the MID-PIWI domain. The spatial arrangement of both lobes differs between the two clades of the Argonaute family: the AGO and the PIWI clade. Since these two clades bind to small RNAs of different sizes (mainly miRNAs and piRNAs respectively), it has been proposed that such spatial arrangement allows them to accommodate small RNAs from different sizes (Matsumoto et al., 2016). The fact that Piwito most probably lacks one complete lobe could reflect a higher permissiveness for small RNAs, not only in terms of length, but also in terms of 3'end recognition.

Interestingly, among prokaryotic Argonaute proteins (pAgos), one subgroup known as short pAgos, comprises genes with the same core structure as Piwito: a PIWI and a MID domain (Burroughs et al., 2013; Hegge et al., 2017). Although the function of short pAgos has not been studied yet, their presence suggests that Argonaute proteins with a 'PIWI-MID only' configuration may be highly evolutionary conserved.

Finally, the N-terminal domain of full-length PIWI proteins has no structural domains but rather regulatory functions. It contains several methylated arginine residues that are necessary for interaction with the PIWI/piRNA pathway components Tudor proteins (Vagin et al., 2009; Mathioudakis et al., 2012). In the germline, Piwil2 interacts with Tudor domain-containing protein 1 (Tdrd1) to ensure efficient repression of transposable elements (Reuter et al., 2009; Wang et al., 2009). Interestingly, in the absence of Tdrd1, Piwil2 was

still loaded with piRNAs, which were however of a different composition compared to the WT scenario. This resulted in repression of different to TE families in the presence or absence or Tdrd1. Thus, Tdrd1 deficiency does not impede piRNA loading or Piwil2 function, but rather influences the final outcome of the silencing machinery (Reuter et al., 2009). Based on these observations it is possible that the lack of an N-terminal domain in Piwito reflects that its regulation and interaction partners may differ from those of full-length PIWI proteins, possibly resulting in the silencing of different TE elements.

7.3.2 *Piwito is regulated by MafB in macrophages*

Our data have also shown that MafB binds the promoter and induces repression of Piwito in non-cycling bone marrow-derived macrophages. This finding may indicate a conserved mechanism for the regulation of PIWI proteins, since the large Maf factor Traffic Jam regulates the PIWI/piRNA pathway in Drosophila gonads (Saito et al., 2009; Robine et al., 2009; Ishizu et al., 2015). Traffic Jam is expressed and regulates Piwi expression in all somatic populations of male and female drosophila gonads (Li et al., 2003; Saito et al., 2009).

Interestingly, MafB deficiency or downregulation renders macrophages more sensitive to cytokine-stimulation in several cellular responses. MafDKO macrophages show a different actin organization (Aziz et al., 2006) and extended proliferation (Aziz et al., 2009) in response to M-CSF. Similarly, MafB-deficient hematopoietic stem cells become more sensitive to M-CSF-instructed myeloid commitment (Sarrazin et al., 2009).

More specifically to the effect in self-renewal, deficiency of MafB (and c-Maf to avoid redundant effects) activates a set of poised macrophage-specific enhancers in genomic loci of self-renewal genes, allowing their expression in response to a cytokine stimulus (Soucie et al., 2016). In this study, we could show that Piwito is regulated in a similar manner. The Piwito locus has activated enhancers in the absence of MafB and its expression can be triggered by M-CSF in Maf-DKO macrophages. Constitutively low MafB levels might enable a similar mechanism in alveolar macrophages and allow induction of Piwito expression upon GM-CSF stimulation.

In addition we have shown that MafB expression increases in microglia during postnatal development, which negatively correlates with the proliferation capacity of these cells. This suggests that low MafB levels in early and pre-microglia may promote self-renewal by allowing the expression of pro-proliferative genes.

7.3.3 *Piwito deficiency limits the lifespan of alveolar macrophages*

Our most striking observation is the fact that Piwito-deficient alveolar macrophages cannot be propagated in long-term culture. This experiment reveals a role for Piwito in alveolar macrophage lifespan. Interestingly, somatic lifespan-control by PIWI proteins seems to be a conserved mechanism, since a recent report also describes a lifespan-regulatory function for the Drosophila Piwi in intestinal stem cells (Sousa-Victor et al., 2017).

A growth disadvantage for Piwito-deficient cells becomes apparent only after several weeks in culture. Our in vivo data shows that Piwito expression is dispensable during homeostasis or for the repopulation of an empty alveolar macrophage niche. Together, these observations suggest that alveolar macrophages in our in vivo experiments did not undergo enough rounds of cell division to become Piwito-dependent. An alternative explanation is that culture conditions induce in the cells more damage or stress than our tested in vivo situations.

Either way, our data suggest that Piwito function is redundant in non-stressed cells that have undergone few divisions. Interestingly, SIRT1 and SIRT6 have been shown to repress repetitive sequences on the transcriptional level in young and healthy mammalian cells. Upon DNA damage or with age, both sirtuins dissociate from the genomic locations of the repetitive elements and re-localize to DNA breaks to aid the local repair machinery. This reorganization is indispensable for DNA repair, but has the side effect that repetitive elements become de-repressed. In both examples, aging or stress are associated with elevated TE activity (Oberdoerffer et al., 2008; Van Meter et al., 2014). It is thus thinkable that in healthy alveolar macrophages that have undergone few divisions, Piwito functions on the repression of TEs are redundant with sirtuins. However, as the cells undergo multiple divisions and accumulate DNA damage in a process of replicative aging, Piwito expression becomes indispensable for self-renewal. Consistent with this hypothesis, a preliminary experiment showed increased levels of LINE1-ORF2 transcripts in long-term cultures of Piwito-KO compared to WT alveolar macrophages, suggesting that retro-transposition may be more active in old alveolar macrophages in the absence of Piwito.

Consistent with the role of SIRT1 in TE silencing, we have shown that knockdown of SIRT1 compromises the self-renewal of macrophages. Although our study revealed that SIRT1 also influences transcriptional pathways relevant to self-renewal and cell cycle control, it is possible that it cooperates with PIWI proteins in TE control, which might lead to partial compensation of Piwito deficiency by SIRT1. Mechanistically, it is possible that Piwito silences TEs post-transcriptionally in the cytoplasm while SIRT1 influences their epigenetic

status. Alternatively, it might be that Piwito and SIRT1 are directed against different families of TEs.

One important question that remains open is why alveolar macrophages and not other macrophage populations express Piwito constitutively, suggesting that they need effective mechanisms for the protection of their genome from TE mobility. Piwito expression in alveolar macrophages may be the combined consequence of constant low MafB levels, sustained self-renewal and the hostile environment of the lung alveoli. As the first line of defense against airway threats, alveolar macrophages are constantly exposed not only to pathogens but also to air pollutants and toxins, which can cause tissue damage (Lowery et al., 2013). Additionally, the lung presents high oxygen levels, which can lead to the production of reactive oxygen species (ROS) and thus oxidative stress (Rogers and Cismowski, 2018). Given the fact that some TE families can be induced by stress (Capy et al., 2000) and that alveolar macrophages are long-lived/self-renewing cells, they may have evolved efficient mechanisms to fight genetic instability.

Transposable elements and PIWI proteins co-evolve at a tremendous speed (Lee 2012). A very illustrative example of this rapid adaptive evolution is given in the ovary of Drosophila: all ovarian somatic cells that surround the germline express the Drosophila Piwi to counteract the effects of TEs. It has been proposed that TEs capable of infecting neighboring cells, such as the retroviral gypsy family, have escaped germline surveillance by shifting their reproductive cycle to adjacent somatic cells. In response to this, somatic cells have evolved a simplified version of the PIWI/piRNA pathway that uses only one PIWI protein. This pathway is simple and direct, has no ping-pong amplification and is directed only against certain families of TEs (Malone et al., 2009; Li et al., 2009). Consistent with this, almost all somatic tissues in Drosophila activate this simplified pathway, including intestinal stem cells in Drosophila where Piwi regulates cellular lifespan. In contrast, the Drosophila germline expresses all PIWI proteins and operates a more elaborated PIWI/piRNA pathway, which functions more effectively and is directed against a broader spectrum of transposable elements. The race to keep transposable elements under control is so desperate, that planarians have evolved an even faster way to be able to control TE activity in different cell types: neoblasts transfer a nuclear PIWI protein to their progeny to ensure TE silencing in those cells, since neoblast progeny, although subjected to TE activity, does not express PIWI proteins by themselves (Shibata et al., 2016).

Adaptive evolution might thus explain Piwito expression in alveolar macrophages that, like somatic cells in Drosophila, might rely on a simplified PIWI /piRNA pathway that utilizes a single PIWI protein. Although a Piwil2/Piwil4-dependent ping-pong mechanism is more efficient in controlling high TE activity, it has been shown that Piwil2 is capable of sustaining an effective piRNA-

dependent TE repression by itself and that cooperation with Piwil4 is not obligatory for the repression of many TE families (Manakov et al., 2015).

Taken into account our observations and the current literature, I propose that, as for the Drosophila soma, a simplified PIWI/piRNA pathway operates in mammalian somatic cells subjected to high levels of TE activity, such as long-lived/self-renewing macrophages. A likely solo player of this pathway is Piwil2 (and its isoform derivates) since its (their) expression has been reported in somatic cells and its ability to act independently of other PIWI proteins has been indicated for the mouse male germline.

7.4 SIGNIFICANCE OF THE STUDY

Here, we report that Piwito, an N-truncated isoform of mouse Piwil2, is necessary to maintain the prolonged lifespan of self-renewing macrophages. This constitutes the first example of a somatic function for PIWI proteins in mammals. Our observations demonstrate that the function of PIWI proteins in the maintenance of long-living, self-renewing cells is not limited to lower invertebrate organisms as previously reported. Furthermore we could show that Piwito regulates the lifespan of macrophages rather than directly affecting cell cycle control. This is consistent with the assumed role in suppression of TE mobility, which is expected to only become detrimental for cellular proliferation after the accumulation of a certain number of cell divisions. In support of this, a similar function of Piwi in lifespan control has been reported for intestinal stem cells in Drosophila (Sousa-Victor et al., 2017), suggesting that lifespan regulation may be a broader function of PIWI proteins.

In the last years, two reports have investigated mammalian PIWI proteins in somatic stem cells but could not detect any functional significance. Using mice deficient for all three mammalian PIWI proteins, the studies reported that PIWI proteins are dispensable for hematopoietic stem cell function (Nolde et al., 2013) and for the generation of functional induced pluripotent stem cells (Cheng et al., 2014). However, the Piwil2 locus of the mice used in both studies carries a deletion of Exon 2-5 (Kuramochi-Miyagawa et al., 2004). This deletion leaves Piwito and other potential short isoforms of the Piwil2 gene intact (Ye et al., 2010; Liu et al., 2017). We hypothesize that a failure to find a phenotype in those studies may be due to the fact that Piwito is still present in the genetic deletion models. With our demonstration of the importance of Piwito for the lifespan of self-renewing cells, it might be warranted to revisit these model systems and investigate whether Piwito also plays a role in murine stem cell self-renewal.

Finally, we have shown that evolutionary conserved mechanisms controlling cellular lifespan in long-lived stem cells of many organisms with high regen-

erative capacity also operate in macrophages, further highlighting the concept that macrophages take advantage of stem cell-like mechanisms to assure self-renewal and long lifespan. We also provided evidence for the regulation of PIWI proteins in mammals: we have shown that the macrophage growth factors M-CSF and GM-CSF can induce the expression of Piwito, whereas the transcription factor MafB can repress it. Regulation of the PIWI/piRNA pathway may thus represent another conserved feature of PIWI proteins, since the only MafB/cMaf ortholog in Drosophila regulates the PIWI/piRNA pathway as well.

7.5 PERSPECTIVES

In the future, it would be interesting to understand how Piwito maintains the lifespan of alveolar macrophages. For this purpose, it is crucial to test whether Piwito can bind and/or cleave piRNAs and whether it is located in the cytoplasm or in the nucleus. In addition, it would be important to test if piRNAs are expressed in alveolar macrophages, and if so, what are their characteristics and potential targets. Another aspect that would require further characterization is the interaction of Piwito with effector proteins. Although Piwito might have the potential to act as the only PIWI protein in the pathway, it may still need cooperation partners for piRNA maturation, piRNA loading or silencing of targets.

Besides investigating the characteristics of the PIWI/piRNA pathway operating in macrophages, it would be interesting to get a clearer idea of the outcome of its activity. To get this information, one could assess whether more retro-transposition, more DNA damage or significant changes in gene expression occur in the absence of Piwito. In alveolar macrophages of young animals, Piwito function appears to be dispensable and might only become critical after alveolar macrophages have undergone many self-renewing divisions. It would therefore be interesting to investigate under which challenges Piwito functions become crucial. One possibility is that Piwito is needed to maintain the fitness of alveolar macrophages upon recurrent viral infections. Indeed, in certain species of vector mosquitoes, PIWI proteins confer tolerance to persistent viral infections. Tolerance, in contrast to resistance, is defined as a defense strategy in which the focus relies on preventing infection-induced damage rather than actively restricting virus growth to clear the infection (Miesen et al., 2016). It is thus possible that Piwito function in vivo becomes crucial in an environment of persistent viral infections or other challenges, such as chronic lung diseases or in aged lungs. Similarly, Piwito might play a role in other situations that increase cellular stress or require high levels of alveolar macrophage proliferation, such as lung injury and subsequent lung regeneration. Possibilities to test these ideas would be to investigate whether Piwito-deficient alveolar macrophages show an early onset of senescence upon genotoxic stress or upon recur-

rent viral infections. Given the role of PIWI proteins in regeneration of lower invertebrates, it would also be very interesting to test whether Piwito-KO mice show impaired lung regeneration.

All mentioned speculations about the function of Piwito carry a relation to aging. Consistent with this notion, aged intestinal stem cells in Drosophila display the same characteristics as young stressed Piwi-KO cells. Remarkably, the ectopic overexpression of Piwi in intestinal stem cells reestablishes cellular fitness in the gut of old flies (Sousa-Victor et al., 2017). In our system, it is thus possible that Piwito-KO alveolar macrophages show an early onset of aging-related cellular decline, which might lead to aberrant or inefficient immune responses (Lowery et al., 2013; Wong et al., 2017).

In conclusion, our data suggest that a function for PIWI proteins in the regulation of cellular lifespan is emerging. Cellular lifespan is maintained by several mechanisms that protect cells from stress and stress-derived accumulation of damage over time. In this sense, PIWI proteins protect the genomes of long-living and/or self-renewing cells by the repression of transposable elements. The effectiveness of PIWI proteins in the maintenance of a genome over time is seen in the cells that depend on PIWI proteins for their long-term preservation: the germline and the somatic cells that drive regeneration in invertebrates, the only known metazoan cell populations carrying an ageless genome.

Among the multiple aspects of cellular decline during aging, a breakdown of genome integrity appears to be one of the most critical aspects for dividing cells. Loss of PIWI function could be thus a critical parameter of aging. Or in other words, sustained expression of PIWI proteins could be a new ingredient to the magic formula for cellular and possibly organismal lifespan extension.

Part IV

APPENDIX

A

SUPPLEMENTARY MATERIAL

Table A1: Antibodies used for Flow Cytometry

Tissue	Antigen, Flurochrome, Clone	Company	Catalog number
Lung or BAL	BV711 anti-CD11c (Clone N418)	Biolegend	117349
	PE anti-SiglecF (Clone E50-2440)	BD Biosciences	552126
	AF647 anti-CD64 (Clone X54-5/7.1)	BD Biosciences	558539
	BV605 anti-CD11b (Clone HL3)	BD Biosciences	563057
	AeF780 anti-F480 (Clone BM8)	eBioscience	47-4801-82
	BV421 anti-Ki-67 (16A8)	Biolegend	652411
Peritoneal Macrophages	FITC AB anti-Ly6C (Clone AL-21)	BD Biosciences	553104
	BV605 anti-CD11b (Clone HL3)	BD Biosciences	563057
	AF647 anti-CD64 (Clone X54-5/7.1)	BD Biosciences	558539
	AeF780 anti-F480 (Clone BM8)	eBioscience	47-4801-82
	PE anti-Tim4 (Clone 54 (RMT4-54))	eBioscience	12-5866-80
	PECy7 anti-B220 (clone RA3-6B2)	Biolegend	103221
	BV421 anti-Ki-67 (16A8)	Biolegend	652411
Microglia	APC anti-CD45.2 (Clone 104)	BD Biosciences	558702
	APCCy7 anti-Ly6C (Clone HK1.4)	Biolegend	128026
	PE-CF594 anti-CD11b (Clone M1/70)	BD Biosciences	562287
MafDKO Cells	PE-CF594 anti-CD11b (Clone M1/70)	BD Biosciences	562287
	BV785 anti-F4/80 (Clone BM8)	Biolegend	123141

Table A2: Primers used for qPCR (Sequence 5′ to 3′)

Target	Forward Primer	Reverse Primer
Target	Forward Primer	Reverse Primer
Piwil1	ATCAGCGTGAAGCCATTGGA	CCACCTCGATCATGATTGCCT
Piwil4	GTCTGCATGACTGGTGCTCT	GAGCCGCACTCTGTTACACT
Piwil2 P1	TTGGCCTCAAGCTCCTAGAC	GAACATGGACACCAAACCTACA
Piwil2 P2	TGCAGCAACACAGATTGCAG	TGACATCCAGCACAGAGTCG
Piwil2 P3	GTCTCTTCCTGCTCGCTGAT	ATAGCATGCATGACATCCAGCAC
Piwil2 P4	GTCAAGGAAGATGACCAGCCG	TCAGCAACATGCCATGGTTATTC
Piwil2 P5	TGGCATGTTGCTGAAGGGCGAG	CACCGTGGTGCTGCTTGGGG
Piwil2 P6	CCATGAAGGACTTGACTCAGCAG	ATTGCTGGCTGTCTCGTTTTG
Piwil2 P7	TGGGAGACTGAAGCAGGAGT	AGGCTTTGGTTTTGTTGTGG
MafB	ACTCCCTGTCCCTGCCATG	CGTCCTTCCTCCCTCTAGCTC
LINE1 ORF2	AGTGCAGAGTTCTATCAGACCTTC	AACCTACTTGGTCAGGATGGATG
HPRT	GGCCCTCTGTGTGCTCAAG	CTGATAAAATCTACAGTCATAGGAATGGA
Puro	TGCAAGAACTCTTCCTCACG	GAGGCCTTCCATCTGTTGC
Actin TSS	GTTCCGAAAGTTGCCTTTTATG	GTACTAGCCACGAGAGAGCGAAG

B

PUBLICATIONS

Matcovitch-Natan, O., Winter, D. R., Giladi, A., Vargas Aguilar, S., Spinrad, A., Sarrazin, S., Ben-Yehuda, H., David, E., Zelada Gonzalez, F., Perrin, P., Keren-Shaul, H., Gury, M., Lara-Astaiso, D., Thaiss, C. A., Cohen, M., Bahar Halpern, K., Baruch, K., Deczkowska, A., Lorenzo-Vivas, E., Itzkovitz, S., Elinav, E., Sieweke, M. H., Schwartz, M. and Amit, I. (2016). Microglia development follows a stepwise program to regulate brain homeostasis, *Science* **353**(6301): aad8670–aad8670. doi: 10.1126/science.aad8670. URL http://www.sciencemag.org/cgi/doi/10.1126/science.aad8670.

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ACRONYMS

3'UTR Three prime untranslated region

AGM aorta-gonad-mesonephros

AGO Argonaute

AKT Protein kinase B (PKB)

AM Alveolar macrophages

AP-1 Activating protein 1

ATAC-Seq Assay for Transposase-Accessible Chromatin using sequencing

AUB Aubergine

BAL Bronchoalveolar lavage

BMDM Bone marrow derived macrophages

BSA Bovine Serum Albumin

CD Cluster of Differentiation

cDNA Complementary DNA

CFA Colony Formation Assay

CFU Colony forming units

ChIP Chromatin immunoprecipitation

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

CSF1R Colony-stimulating factor 1 receptor

DAMPs Danger-associated molecular patterns

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethylsulfoxid

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

EdU 5-ethynyl-2 deoxyuridine

EGTA Egtazic acid

EMP Erythromyeloid progenitors

ERK Extracellular signal-regulated kinases

ESCs Embryonic stem cells

EV Empty vector

FACS Fluorescence-activated cell sorting

FCS Fetal calf serum

gDNA Genomic DNA

GFP Green fluorescent protein

GM-CSF Granulocyte-macrophage colony-stimulating factor (Csf2)

GM-CSFR Granulocyte-macrophage colony-stimulating factor receptor (Csf2r)

GSC Germ stem cell

HBSS Hank's Balanced Salt Solution

HEPES 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfon acid

HP1 Heterochromatin protein 1

HPRT Hypoxanthin-Guanin-Phosphoribosyltransferase

HSC Hematopoietic stem cell

HSF-1 Heat shock factor 1

IAP Intracisternal A Particle

IFN γ Interferon-γ

IGF-1 Insulin-like growth factor 1

IL Interleukin

IL-4Rα IL-4 receptor alpha chain

iNOS Inducible nitric oxide synthase

iPS cells Induced pluripotent stem cells

JAK Janus kinase

KD Knockdown

Klf2 Kruppel-like factor 2

Klf4 Kruppel-like factor 4

KO Knockout

LAD Lamin-associated domain

LINE-1 Long-interspersed nuclear element 1

LPS Lipopolysaccharide

LTR Long terminal repeats

MafDKO MafB, cMaf Double Deficiency

MAPK Mitogen-activated protein kinase

MARE Maf recognition element

M-CSF Macrophage Colony-stimulating factor (Csf1)

M-CSFR Macrophage colony-stimulating factor receptor (Csf1r)

MID Middle domain

miRNAs Micro RNAs

mRNA Messenger RNA

mTOR Mammalian target of rapamycin

NAD+ Nicotinamide adenine dinucleotide

NAM Nicotinamide

NHP Non-human primate

NMD Nonsense-mediated decay

NOD Nucleotide-binding oligomerization domain

NOS Nitric oxide synthase

N-PAZ PIWI-ARGONAUTE-ZWILLE

ORF Open reading frame

PAMP Pathogen-Associated Molecular pattern

PAP Pulmonary alveolar proteinosis

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PDGF Platelet-derived growth factor

PDL1 Programmed death ligand 1

PDL2 Programmed death ligand 2

PGCs Primordial germ cells

PI3K Phosphoinositide 3-kinase

piRNA PIWI-interacting RNA

PIWI P-element induced wimpy testis

PIwil1 Piwi-like 1

PIwil2 Piwi-like 2

PIwil4 Piwi-like 4

PM Peritoneal macrophages

PPAR-γ Peroxisome proliferator-activated receptor gamma

qPCR Quantitative polymerase chain reaction

RELMα Resistin-like molecule-α

RNA Ribonucleic acid

ROS Reactive oxygen species

RPMI Roswell Park Memorial Institute medium

RT-qPCR Reverse transcription qPCR

rtTA Reverse tetracycline-controlled transactivator

SFK Src family kinases

shRNA Small hairpin RNA

SINE Short Interspersed Nuclear Element

SIRT Sirtuin

SSCs Spermatogonial stem cells

STAT Signal Transducer and Activator of Transcription

STS Stauro sporine

TE Transposable element

TET Tetracycline

TGF-β1 Transforming growth factor β1

TJ Trafic jam

TNFα Tumor necrosis factor alpha

TSS Transcription starting site

VEGF Vascular endothelial growth factor

WT Wild type

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